INVESTIGATIONS ON CONFORMATIONAL PROPERTIES, INTERACTIONS WITH SMALL MOLECULES, AND MEMBRANE ACTIVITIES OF SYNTHETIC CYCLIC PEPTIDES

SHUNSAKU KIMURA

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Department of Polymer Chemistry
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LIST OF ABBREVIATIONS

The abbreviations used in this thesis are listed below.

Abbreviations of Reagents

DCCI	Dicyclohexylcarbodiimide
HOSu	N-Hydroxysuccinimide
HOBt	1-Hydroxybenzotriazole
TFA	Trifluoroacetic acid
DMSO-d_6	Dimethyl sulfoxide-d_6
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Tris	Tris(hydroxymethyl)aminomethane
ANS	Sodium 8-anilino-1-naphthalenesulfonate
DNS-Cl	Dansyl (5-(dimethylamino)naphthalene-1-sulfonyl) chloride
DPPC	Dipalmitoylphosphatidylcholine
EtOH	Ethanol
Diox	Dioxane
AcOEt	Ethyl acetate
Yb(fod)$_3$	Tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl 3,5-octanedionato)ytterbium

Abbreviations of Protecting Groups of Peptides or Functional Groups

Boc	t-Butyloxycarbonyl
Z	Benzyloxycarbonyl
OMe	Methyl ester
OEt	Ethyl ester
OBzl	Benzyl ester
OSu or OSuc	N-Hydroxysuccinimide ester

Bzl(OMe)	p-Methoxybenzyl
DNS	Dansyl

Other Abbreviations

TLC	Thin layer chromatography
R_f(I)	Rate of flow of the material subjected to TLC in $CHCl_3/CH_3OH/CH_3COOH$(95/5/3)
R_f(II)	Rate of flow of the material subjected to TLC in 1-butanol/CH_3COOH/H_2O/pyridine (30/6/24/20)
R_f(III)	Rate of flow of the material subjected to TLC in $CHCl_3/CH_3OH$/pyridine(95/5/3)
R_f(IV)	Rate of flow of the material subjected to TLC in 1-butanol/CH_3COOH/H_2O(65/10/25)
R_f(V)	Rate of flow of the material subjected to TLC in ethyl acetate/CH_3OH(4/1)
ANAL.	Elementary analysis
GPC	Gel permeation chromatography
FDMS	Field desorption mass spectrum
m.p.	Melting point
M	$Mol \cdot l^{-1}$
RT.	Room temperature
nmr	Nuclear magnetic resonance
cd	Circular dichroism
UV	Ultraviolet
ir	Infrared

List of the Spectroscopes Used in This Thesis

UV	Shimadzu UV-210 spectrophotometer
cd	JASCO J-20 spectropolarimeter
90 MHz ^{1}H and ^{13}C nmr	JEOL FX90Q Fourier transform nmr spectrometer
100 MHz nmr	Varian HA100 spectrometer
270 MHz nmr	Brücker WH270 Fourier transform nmr spectrometer
fluorescence	Hitachi MPF-4 spectrofluorometer

INTRODUCTION

Recently much attention and interest have been focused on membrane systems which include biomembranes as well as artificial membranes. The role of biomembrane, which is the regular assembly of lipid molecules, is not only a barrier of living cell but also a highly functional unit for life phenomena. The membrane functionalities are based upon specific interactions between membrane proteins and lipid molecules. With respect to artificial membranes, ion-exchange membrane, ultrafiltration membrane, and reverse osmosis membrane have been developed, and new types of artificial membranes such as ion-selective membrane and artificial kidney are being investigated. For the latter membranes to be realized, materials having higher-order chemical function as well as biological function should be developed. Therefore, the clarification of the function of biomembrane is useful not only to understand the mechanism of life phenomena but also to acquire valuable information to develop the highly functionalized membranes.

Since the interior of biological lipid membrane is hydrophobic, the permeation of ionic substances through membrane is generally restricted. The concentration distibution of ionic species across the membrane is closely related to life phenomena. For example, the concentration gradient of proton across mitochondrial membranes and chloroplasts is a driving force to generate " high energy intermediate "(1). It is well-known that different concentrations of Na^+ and K^+ between the inside and the outside of cell membrane is the basis of cell activities(2). Furthermore, Ca^{2+} ions play an important

role in the contraction of muscle(3), the release of neurotransmitter at synapse(4), and the release of cyclic AMP(5). It is assumed that membrane proteins are oriented asymmetrically with respect to the plane of membrane as a result of the interaction between charge cluster of the membrane proteins and surface potential of the membrane. The asymmetric orientation of membrane proteins is arisen from the asymmetric distribution of Ca^{2+} ion across the membrane(6). Therefore, if the ion permeability through membrane is controlled at will, a lot of information about physiological phenomena will be obtained, which will in turn contribute as the basis to develop useful functional materials.

A group of compounds is classified as ionophore which can transport ionic species selectively through lipid membrane. The mechanism of transport is explained in terms of the following successive steps; i) ion binding at one side of the membrane, ii) hydrophobic complex diffusing through the membrane, iii) release of the ion at the other side of the membrane. However, the behavior of the ion carrier at the water/membrane interface or in the interior of membrane has not been unambiguously understood on a molecular level. For instance, macrolide actin, valinomycin, and dicyclohexyl-18-crown-6 have been shown to transport K^+ ion through mitochondrial membrane, and a rough proportionality has been observed between the transport rate and the ability of ion extraction by the ionophores from aqueous phase into organic phase. However, the ratio of the transport rate against the extraction coefficient differs from an ionophore to the others, that is, valinomycin transports K^+ ion in a rate 1/10 as slow as that expected from its extraction coefficient relative to macrolide actin.

The ion transport by dicyclohexyl-18-crown-6 is about 1/1000 as slow as that predicted from the extraction coefficient(7). In order to explain the discrepancy, the non-uniform distribution of carrier molecule over the membrane and the easiness of the interconversion from a hydrophilic complex formed at the water/membrane interface into a hydrophobic complex suitable to diffuse through the interior of membrane have been discussed. Furthermore, the ion selectivity by ionophores has been shown to depend on the solvent. For example, the transport ratio of K^+/Rb^+ by valinomycin has been shown to depend very much on the solvent(8). This fact indicates that the formation of ion complex by ionophore is greatly influenced by the nature of environment. Lipid membranes are characterized by a hydrophobic region in contact with a hydrophilic region, which is quite different from homogeneous solution. Therefore, the complexation in membrane should be investigated in a real membrane system and compared with that in homogeneous solution.

The investigation on the ionophore/membrane interaction using a model system is quite useful to analyze the membrane activities on a molecular level, because with the model system we can relate the molecular function to the structure. Naturally-occurring ionophores such as valinomycin, enniatin, and antamanide are cyclic depsipeptide or cyclic peptide. Even linear ionophores such as X537A and nigericin take a cyclic structure in the complex formation with ion(9). From these facts cyclic peptides were considered to be very suited as model compounds for ionophore. Cyclic peptides have the following advantages; i) the number of available conformations is much reduced by cyclization to enable the structural analysis by various spectroscopic methods,

ii) carbonyl groups of the cyclic skeleton may cooperate to bind cations, iii) various functional groups can be incorporated into the molecule by a proper choice of naturally-occurring amino acids(10). From these points of view, a series of cyclic peptides was synthesized in this thesis. The conformation, the complex formation, and the specific interactions in lipid membranes of those cyclic peptides were investigated.

In Part I of this thesis, a number of cyclic penta, cyclic hexa, and cyclic octapeptides have been synthesized for the first time, and their conformational properties in solution were investigated. While usual peptide bonds take a trans planar structure, N-substituted peptide bonds take cis as well as trans form. Therefore, the conformation of cyclic peptides containing N-substituted amino acids is versatile. The lack of intramolecular hydrogen bonding increases their segmental mobility and the lipophilicity of N-substituted peptides. In Chapter 1, a cyclic hexapeptide, cyclo(Pro-Sar-Sar)$_2$, which consists of N-substituted amino acids only, was synthesized and its conformation in solution was investigated. This cyclic hexapeptide was shown to take various conformations arising from the cis/trans isomerization of peptide bonds. Therefore, this cyclic peptide is flexible in a thermodynamic sense. In addition, the internal rotations around the single bonds of cyclic skeleton were suggested to be rapid on the nmr time scale. Therefore, this cyclic peptide is flexible in a dynamic sense, too.

Cyclic peptide containing a tryptophan residue has a fluorescent indolyl group in side chain, and its conformation and interaction can be studied by the fluorescence method. However, since the indolyl group

is easily oxidized or damaged by side reactions, this kind of cyclic peptide has been seldom synthesized. In Chapter 2, cyclic pentapeptide containing a tryptophan residue, cyclo(Try-Sar-Sar-Lys(DNS)-Pro), was synthesized and its conformation in solution was investigated. This cyclic pentapeptide was found to contain one cis peptide bond, and is apt to take a β-turn structure in which Pro locates at the 3rd position. This type of β-turn structure is very unique for L-X-L-Pro sequence.

When the arrangement of carbonyl groups of cyclic skeleton is highly symmetrical, an efficient and selective cation binding is expected by the intramolecular cooperation of the carbonyl groups. In Chapter 3, a series of cyclic octapeptides cyclo(X-Pro)$_4$, in which X represents Phe, Leu or Lys(Z) residue and a symmetric structure is expected, was synthesized and the conformation in solution was investigated. The number of available conformations of these cyclic octapeptides varied according to the nature of X residue, which suggests that the steric repulsion between side chains of component amino acid residues greatly affects the conformational distribution of cyclic skeleton. These cyclic octapeptides in a free state are apt to take a C_2-symmetric conformation containing a β-turn structure in which Pro residue locates at the 3rd position, which is very unique for L-X-L-Pro sequence. The conformational distributions of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ in a free state depend sharply on solvents, and in certain kinds of solvents they take a highly symmetric C_4-symmetric conformation.

In Part II of this thesis, the complex formation of cyclic peptides with small molecules such as metal ions and α-amino acid ester salts were investigated in

relation to their conformational properties. In Chapter 4, bis cyclic dipeptides, in which two cyclic dipeptide moieties are connected by the bridge of various lengths and properties, were investigated. It was suggested that by making a proper choice of the nature of the bridge, a certain ion selectivity could be realized. In Chapter 5, the complexation of metal ions and ammonium salts by cyclo(Pro-Sar-Sar)$_2$, which is flexible in either thermodynamic or kinetic sense, was investigated. This flexible cyclic peptide formed complexes with several metal ions accompanied by the conformational change which was dependent on the nature of metal ions. Therefore, the flexible cyclic peptide was an excellent ion binder but its ion selectivity was low. In Chapter 6, the complexation of metal ions by cyclic octapeptides, cyclo(Phe-Pro)$_4$, cyclo(Leu-Pro)$_4$, and cyclo(Lys(Z)-Pro)$_4$, were investigated. Cyclo(Phe-Pro)$_4$, which consisted of the only C_2-symmetric conformation in a free state, showed a very poor ability of complexation. On the other hand, cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ were shown to form complex with several metal ions and selectively with Ba^{2+} ion. In the complexed state they took a C_4-symmetric conformation. The ion selectivity was high and determined by the fit of Ba^{2+} ion to the hydrophilic cavity formed by four carbonyl groups of cyclic octapeptide. The shape and the size of the hydrophilic cavity are determined by the steric repulsion among side chains of the amino acid residues. This conclusion is a useful guide to design highly selective ion binders.

In Part III of this thesis, the ion transport through an organic liquid membrane by the cyclic octapeptides and the interactions of linear and cyclic

peptides in lipid membrane were investigated. In Chapter 7, the ion transport through chloroform membrane by the cyclic octapeptides were investigated. Cyclo(Leu-Pro)$_4$ was shown to be an efficient ion carrier, and cyclo-(Leu-Pro)$_4$ transported K^+ ion 1/3 times as fast as that by dicyclohexyl-18-crown-6, which has been shown to be the most efficient K^+-carrier among crown ethers. The picrate-anion transport by cyclo(Leu-Pro)$_4$ against its concentration gradient was observed. The anion transport was coupled with the selective transport of K^+. Though the cyclic octapeptides transported ions through chloroform membrane efficiently and selectively, they did not work as an efficient ionophore in lipid membrane. Therefore, the basic aspects of behaviors of peptides in lipid membrane were investigated further in details. Fluorescence method was mainly used for the investigations in membrane, because the measurement of fluorescence can be carried out with a small amount of sample, so that the perturbation of membrane structure caused by the sample may be minimized. Furthermore, the fluorescence method has an advantage to get various kinds of information from the fluorescence spectra, polarization of fluorescence, the life time of excited state, and the energy transfer, etc. In Chapter 8, the peptide-lipid and the peptide-peptide interactions in lipid membrane were studied by measuring the excited energy transfer from linear peptides containing a tryptophan residue to lipids or other peptides having an energy-acceptor group. As a result, it was suggested that the phase transition of the membrane greatly affected the distribution of the probes in membrane. That is, a phase separation in membrane was induced by the phase transition to produce crystalline regions excluding the

probes as well as domains containing high concentrations of the probes. A specific interaction was suggested to occur between donor peptide and acceptor peptide. In Chapter 9, the intramolecular energy transfer in the cyclic pentapeptide containing an energy donor and an energy acceptor group was investigated to get basic knowledge on photo energy conversion in membranes. The intramolecular energy transfer was affected by the pre-transition of the membrane. This observation indicates a possibility that the conformation of membrane peptide can be regulated by the structural change of membrane related to the phase transition. The intramolecular energy transfer of the cyclic peptide changed by the complex formation with Ba^{2+} ion. This is a very promising result to indicate a control of energy transfer by ion binding.

The present investigation using various cyclic peptides clarified many aspects of the membrane activities of peptides in relation to the conformational properties, and presented several important guides to design membrane active peptides. The experiments using fluorescent peptides revealed various aspects of interactions of peptides in lipid membrane. In addition, the model systems revealed that the intramolecular energy transfer involving a peptide was controlled by the structural change of the lipid membrane or the complex formation of the peptide. These instances are the valuable clue to develop a multi-functional peptide/membrane composite system, in which a certain function is manifested under the control of the other functions.

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PART I

Synthesis and Conformation of Cyclic Peptides

Chapter 1

Synthesis and Conformation of Cyclic Hexapeptide Cyclo(Pro-Sar-Sar)$_2$

INTRODUCTION

Investigations on the complexation by cyclodextrins and crown ethers as models for the molecular recognition by enzymes have become very popular, and the host-guest chemistry has become an important research field(1). This field is related not only to the selective binding of a substrate by macromolecules but also to all kinds of life phenomena such as the hormone-receptor adaptation. In this regard, cyclic peptides seem to be a useful probe to investigate the molecular recognition. Cyclic peptides have been used as a structural model of proteins for conformational analysis, because they can reconstruct conformational characteristics unambiguously. On the other hand, some of synthetic cyclic peptides have been found to complex with alkali metal ions or ammonium ions by the cooperative action of peptide carbonyl groups(2). Functions of cyclic peptides such as complexation with low molecular weight compounds can be investigated in connection with the conformational properties, and this line of investigation may provide useful information on molecular recognition by biopolymers(3,4).

There are many biologically active cyclic peptides and cyclic depsipeptides, known as ionophores(5), in which N-substituted amino acids are often involved. N-substituted peptide groups enhance the solubility of the peptide in organic solvents, produce cis as well as trans conformation, and eliminate hydrogen bonding with peptide

carbonyl groups. A series of cyclic hexapeptides containing sarcosine and proline has been synthesized and investigated(6-8). As a consequence, it has been found that the N-substituted amino acid residue increases the numbers of available conformations, and that cyclic hexapeptides which can take a variety of conformations bind alkali metal ions effectively. It was predicted that cyclo(Pro-Sar-Sar)$_2$, which is a cyclic hexapeptide consisting only of sarcosine and proline, is flexible enough to take many different conformations, soluble in organic solvents, and capable of binding metal ions. Therefore, cyclo(Pro-Sar-Sar)$_2$ was expected to be a suitable model for ionophores.

In this chapter, the synthesis of cyclo(Pro-Sar-Sar)$_2$, conformational analysis using 270 MHz nmr, and the conformational properties found from calculation using a hard-sphere model are described.

EXPERIMENTAL

Synthesis of Cyclo(Pro-Sar-Sar)$_2$

The synthetic route is shown in Figure 1. The peptide chain was elongated through the N-terminal by the usual procedure using DCCI as a condensing agent, to give a protected linear tripeptide, Boc-Sar-Sar-Pro-OBzl.

Boc-Sar-Sar-Pro-OH

Boc-Sar-Sar-Pro-OBzl was subjected to catalytic hydrogenation by Pd/black in methylene chloride. After filtering off the catalyst, the filtrate was evaporated under reduced pressure. The residual oil solidified on treatment with ethyl acetate/n-hexane.

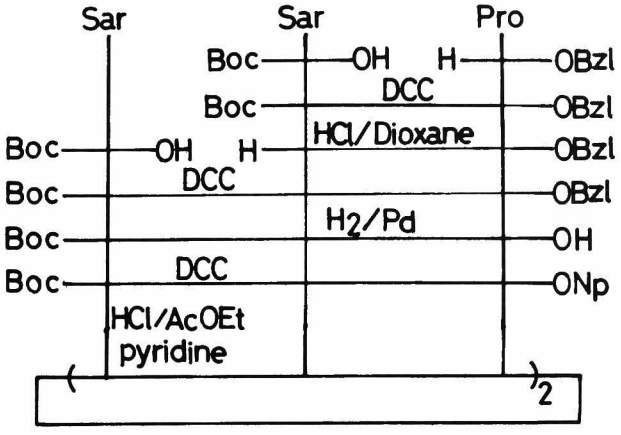

Fig. 1. Synthetic route of cyclo(Pro-Sar-Sar)$_2$.

The white mass was recrystallized from ethyl acetate. The yield was 72% on the basis of HCl·Sar-Pro-OBzl. ANAL. Calcd. for $C_{16}H_{27}O_6N_3$: C, 53.77%; H, 7.61%; N, 11.76%. Found: C, 53.92%; H, 7.74%; N, 11.62%.

Boc-Sar-Sar-Pro-ONp

To a methylene chloride solution of Boc-Sar-Sar-Pro-OH (6.00g) and p-nitrophenol (3.04g), DCCI (4.51g) was added at 0°C. After stirring for 3 hours at 0°C, the solution was left standing overnight at room temperature. After filtering off insoluble dicyclohexylurea , the filtrate was evaporated under reduced pressure. The residue was extracted with ethyl acetate and again dicyclohexylurea was filtered off. Ether was added to the filtrate. A pale yellow product, precipitated by cooling the mixture, was quickly filtered and dried. Yield 70%.

Cyclo(Pro-Sar-Sar)$_2$

To a solution of Boc-Sar-Sar-Pro-ONp (5.0g) in ethyl acetate (30ml), ethyl acetate (60ml) containing 3.2 N HCl was added at 0°C. After stirring for 40 min, petroleum ether was added and the white precipitate was quickly filtered and dried over NaOH. The product was dissolved in dimethylformamide (80ml) containing acetic acid (0.2 ml), and the solution was added dropwise to pyridine (1300ml) at 60°C. The solvent was evaporated off under vacuum, and the residual oil was dissolved in methanol–water (1:1). The solution was eluted through IR 45 and IR 120B columns and the eluted solution was condensed in vacuum. The residual yellow oil was dissolved in methanol and fractionated on a Sephadex LH-20 column. Fractions containing a ninhydrin-negative component having R_f(II) = 0.30 on TLC were collected and evaporated. A pale yellow solid was obtained which was dissolved in hot tetrahydrofuran and reprecipitated by adding ether. The white solid obtained was recrystallized from methanol/ether mixture: yield 6%; m.p.>300°C; m/e in mass spectrum 478 (theoretical 478). ANAL. Calcd. for $C_{22}H_{34}O_6N_6 \cdot 1/2H_2O$: C, 54.20%; H, 7.24%; N, 17.24%. Found: C, 53.97%; H, 6.94%; N, 17.18%.

Procedure

Nmr spectra were recorded using a Brücker WH 270 type Fourier transform spectrometer, courtesy of Professor T.Miyazawa, University of Tokyo. Tetramethylsilane was used as a reference compound.

Conformational calculation was made using FACOM M-190.

RESULTS

Solution Conformation of Cyclo(Pro-Sar-Sar)$_2$ Investigated by Circular Dichroism and Nuclear Magnetic Resonance Spectroscopy

270 MHz nmr spectra of cyclo(Pro-Sar-Sar)$_2$, which is hereafter abbreviated to c(PSS)$_2$, are shown in Figure 2. A series of strong signals (a) appearing at ~ 3 ppm is ascribed to Sar N-CH_3 protons on the basis of their high intensity and the comparison with other cyclic peptid containing sarcosine. There are many Sar N-CH_3 signals, indicating the existence of many kinds of conformation. The comparison of the spectra in D_2O and $CDCl_3$ indicates that the conformational distribution depends on the nature of the solvent and more conformations are available in more polar solvents. Many of the conformers evidenced by nmr spectroscopy are likely to be the cis/trans isomers of peptide bonds.

In the nmr spectra, the Sar N-CH_3 proton signals are divided into two groups. When a small amount of benzene was added to the chloroform solution of c(PSS)$_2$, the N-CH_3 signals at lower magnetic field shifted to higher magnetic field to a greater extent than those already at higher magnetic field (see Figure 3). On the basis of information on the effect of benzene(7), the signals appearing at lower magnetic field are ascribed to Sar N-CH_3 protons adjacent to a trans peptide bond, and those at higher magnetic field to those to a cis peptide bond.

If c(PSS)$_2$ takes a C_2-symmetric conformation, N-CH_3 protons of four Sar residues should give two signals having equal intensity. Therefore, the available

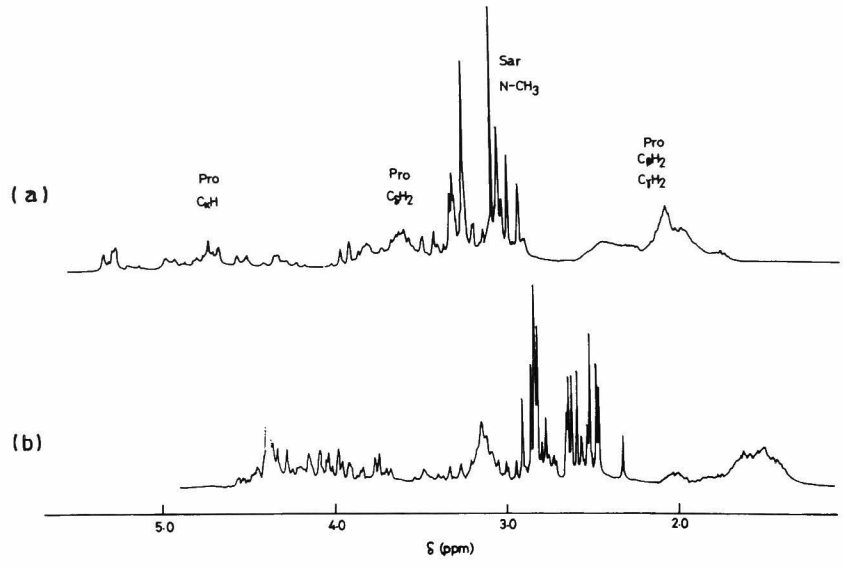

Fig. 2. 270 MHz nmr spectra of cyclo(Pro-Sar-Sar)$_2$. (a), $CDCl_3$ solvent, c(PSS)$_2$; 29.4 mM. (b), D_2O solvent, c(PSS)$_2$; 26.0 mM.

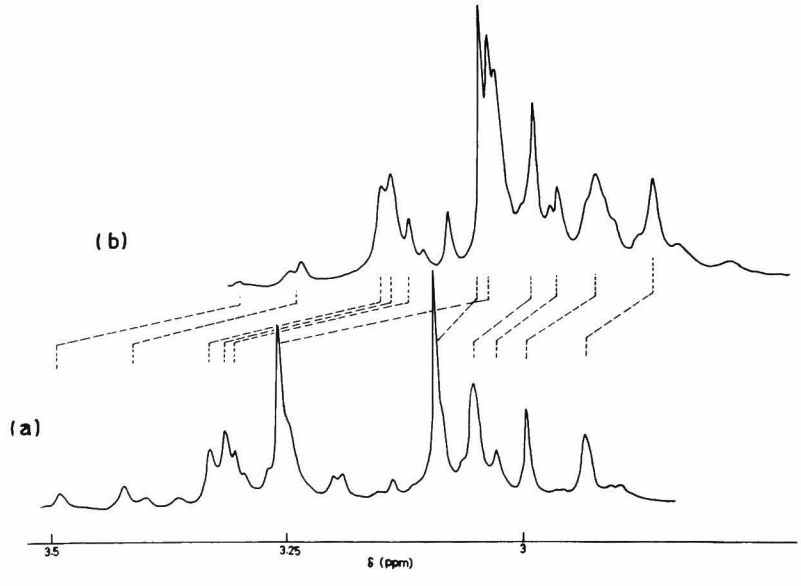

Fig. 3. Expanded N-CH_3 signals of 270 MHz nmr spectra of cyclo(Pro-Sar-Sar)$_2$. (a), $CDCl_3$ solvent; (b), $CDCl_3/C_6D_6$ solvent (4:1).

conformations were investigated by sorting the N-CH_3 signals into several pairs of two or four signals having equal intensity. Figure 4 shows the nmr spectra of c(PSS)$_2$ in $CDCl_3/C_2D_5OD$ mixture in which the solvent composition was varied continuously. In any of the mixed solvents Sar N-CH_3 signals are split into two groups, and the signals appearing at lower magnetic field were ascribed to N-CH_3 protons adjacent to a trans peptide bond and those at higher magnetic field to those adjacent to a cis peptide bond. For comparison, a broken line and a dotted line in Figure 5 represent the chemical shift changes of methylene and methyl protons of undeuterated ethanol, respectively, for which the ordinate is conveniently shifted. It is seen in Figure 5 that the signal marked x, which appears at the lowest magnetic field in $C_2D_5OD/CDCl_3$ (5:95 v/v), does not exist in pure chloroform and is evident only when ethanol is added. The spectral change induced by the addition of 5% C_2D_5OD is more marked than for other cases. This observation suggests that the conformational distribution of c(PSS)$_2$ is greatly influenced by the specific solvation by ethanol which forms hydrogen bond.

A pair of two strong signals (circles in Figure 5) in $CDCl_3$ were ascribed to a C_2-symmetric conformation. In this conformation the four peptide bonds involving sarcosine residue P_1, P_3, P_4, P_6 should take either cis-trans-cis-trans or trans-cis-trans-cis sequence. Other N-CH_3 signals were classified into several pairs in the same way (see Figure 5). The characteristics of these pairs of signals are listed in Table I. Consequently, seven kinds of C_2-symmetric conformations were detected, which are distinguishable on the nmr time scale.

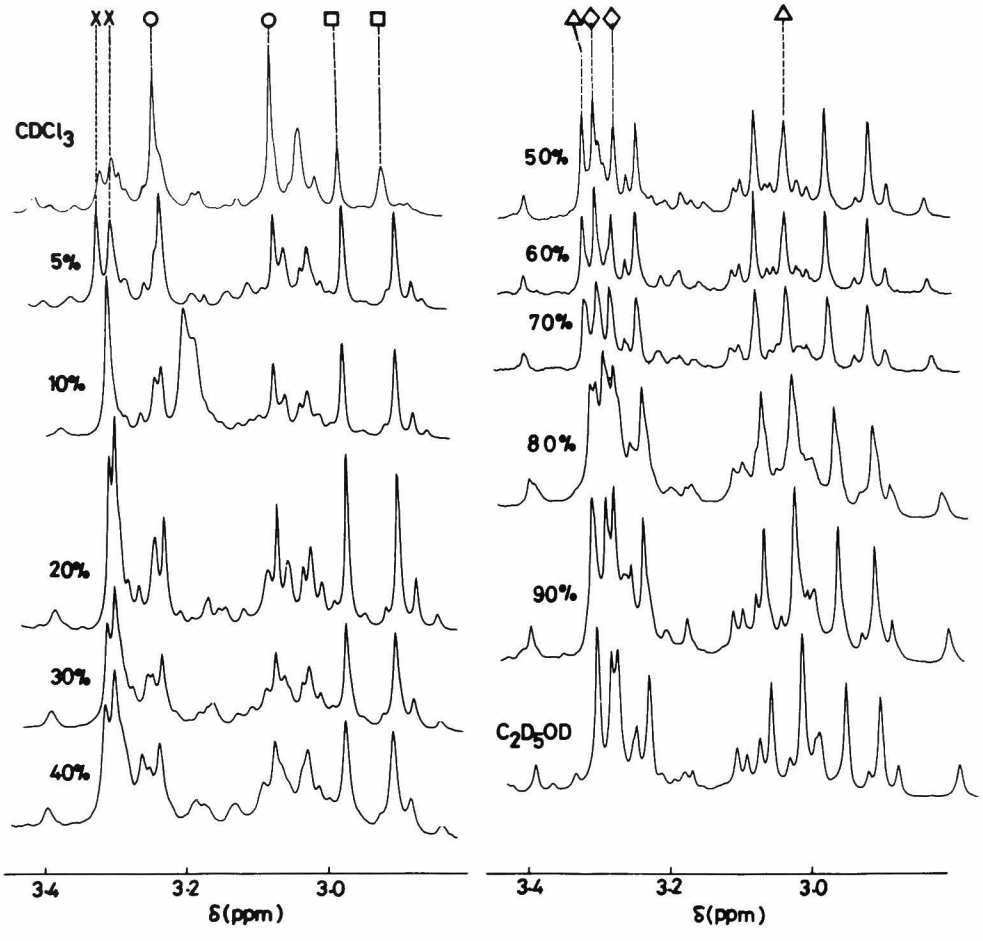

Fig. 4. Change in N-CH_3 signals of cyclo(Pro-Sar-Sar)$_2$ due to solvent composition. Numbers indicate the vol% of C_2D_5OD. o-o, □-□, x-x, △-△, and ◇-◇ represent the pairs of signals ascribable to C_2-symmetric conformations.

Conformation of Cyclo(Pro-Sar-Sar)$_2$ as Studied by the Calculation using a Hard-Sphere Model

Spatially allowed conformations of c(PSS)$_2$ were calculated using a hard-sphere model. With bond lengths and bond angles being fixed, the linear hexapeptide fragment shown in Figure 6 was generated in a computer. Among linear hexapeptides corresponding to various dihedral angles, those having both ends within a certain distance without the skeletal overlapping were counted. Only the C_2-symmetric conformations were computed in the present investigation. Therefore, the independent variables are the peptide bonds P_1, P_2, and P_3 and the dihedral angles ϕ_p, ψ_p, ϕ_s, ψ_s, and ψ'. 0° or 180° was given to P_1, P_2 and P_3, and ϕ_p, ψ_p, ϕ_s, and ψ_s were varied by 10° between -180° and 180°. For ψ' the values reported

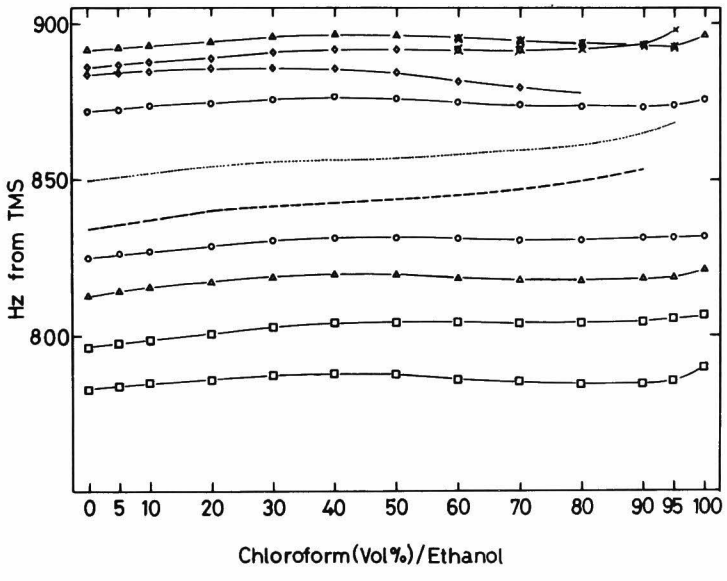

Fig. 5. Dependence of the chemical shifts of N-CH_3 signals of cyclo(Pro-Sar-Sar)$_2$ on the solvent composition. Symbols correspond to those in Figure 4. Chemical shifts of CH_3 (·····) and CH_2 (---) signals of ethanol are also shown.

TABLE I

Conformation of Cyclo(Pro-Sar-Sar)$_2$ by Nmr Spectroscopy

Nature of peptide bond [a]		
α	β	Observations
C	T (or T, C)	Major conformation (36%) in $CDCl_3$
T	C (or C, T)	Major conformation (20%) in C_2D_5OD
C	C	Present in any solvent composition
T	T	Prevails in 5-40 vol% C_2D_5OD
T	T	Prevails in 30-100 vol% C_2D_5OD
C	T (or T, C)	Minor conformation
T	C (or C, T)	Minor conformation

a) cyclo(Pro$^{\alpha}$-Sar$^{\beta}$-Sar)$_2$: C, cis; T, trans

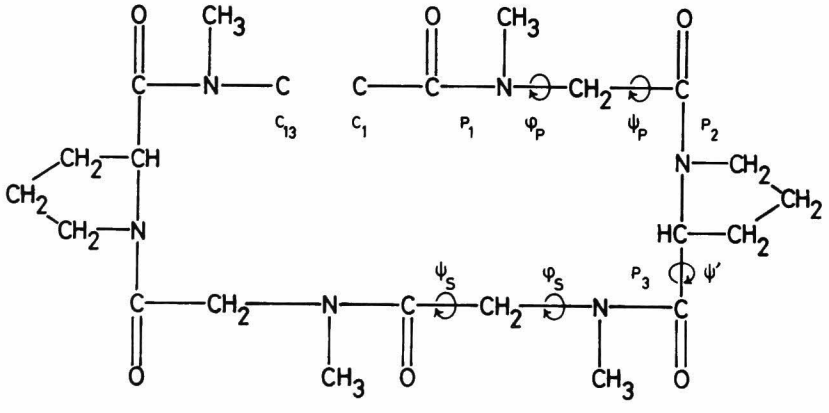

Fig. 6. Linear hexapeptide for the calculation. For the condition of parameters, P_1, P_2, P_3, ϕ_p, ϕ_s, ψ_p, ψ_s, and ψ', see the text.

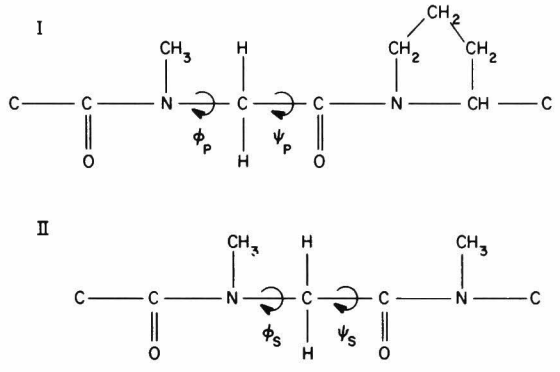

Fig. 7. Element dipeptides, Sar-Pro (I) and Sar-Sar (II).

for acetylproline dimethylamide were used(9), which were varied by 10° between 105° and 185° for trans P_2 and between 135° and 185° for cis P_2. The linear hexapeptide was divided into two fragments, I and II (see Figure 7), and the range of dihedral angles allowed for each linear dipeptide was determined. Allowed dihedral angles were then combined to generate linear hexapeptides. Due to the strain of cyclic peptides, the bond length and the bond angle may deviate from the usual values allowed for linear peptides. Therefore, the geometry of the proline residue was so determined that the allowed region of dipeptide map is as wide as possible. For bond angles of the fragment peptide I, the values proposed by Scheraga were used (10), except $\angle C^{\alpha}C'N$ and $\angle C^{\alpha}C'O$. For the latters, the values of the naturally-occurring cyclic peptides actinomycin(11) and antamanide(12) were adapted. For the sarcosine residue, the values reported on the bases of X-ray diffraction studies(13) were used. The atomic radius was 1.6 Å for methyl group and the values reported by Scott and coworkers

(14) were used for other atomic radii.

The production of linear chains in which terminal atoms C_1 and C_{13} come within 4 Å is shown in Table II. When the distance R between C_1 and C_{13} is longer, the cyclic conformation in which C_1 and C_{13} overlap should be more strained and less probable. Table II shows that the conformers having R<2.5 Å are possible when the conformational sequence of peptide bonds $P_1P_2P_3$ is TTT,TTC, CTT, or CCT. Conformers having TCT or CTC sequence of peptide bonds are possible only when R is taken to be 4 Å. However, these conformations are not likely, because the Corey-Pauling-Koltun molecular models of the cyclic peptides under the conditions determined were found to suffer a severe atomic overlapping and it is not relieved with suitably modified dihedral angles. In conclusion, the computer calculation predicted that four C_2-symmetric conformations having R<2.5 Å and TTT,TTC,CTT,and CCT sequences of peptide bonds would be possible.

DISCUSSION

As expected, the solubility of $c(PSS)_2$ in organic solvents was high. This property must have come from the presence of proline residues and the absence of amide hydrogen. The latter factor implies that the molecular skeleton of $c(PSS)_2$ is flexible compared with other cyclic peptides that involve intramolecular hydrogen bonding by amide groups. The properties of $c(PSS)_2$ showed that there are many conformational isomers which are distinguishable from each other on the nmr time scale (due to cis/trans isomerization around peptide bonds), and that the conformational distribution changed according to the ability of the solvent to form hydrogen bonds. Thus,

TABLE II

Conformation of Cyclo(Pro-Sar-Sar)$_2$ by Calculation

Nature of peptide bond[a] $P_1P_2P_3$	Nature of dipeptide conformation[b]	Number of total chains[c] $R \leq 4$ Å	Number of chains after atomic overlap check[c]					
			R=0-1	1-1.5	1.5-2	2-2.5	2.5-3	0-4 Å
T T T	P S	158	-	4	10	16	30	158
	P S	16	-	-	-	-	3	16
	P S	10	-	-	-	-	-	10
T T C	P S	650	-	2	1	8	7	50
T C T	P S	271	-	-	-	-	-	21
T C C	P S	39	-	-	-	-	-	-
	P S	4	-	-	-	-	-	-
	P S	18	-	-	-	-	-	-
C T T	P S	701	-	-	2	6	29	188
	P S	4	-	-	-	-	-	-
	P S	162	-	-	-	-	-	-
C T C	P S	456	-	-	-	-	1	3
	P S	45	-	-	-	-	-	-
C C T	P S	426	-	-	-	4	1	34
	P S	26	-	-	-	-	-	-
C C C	P S	96	-	-	-	-	-	-
	P S	2	-	-	-	-	-	-

a) T, trans; C, cis.

b) PS, allowed region on the dipeptide map corresponding to $0^\circ<\phi<180^\circ$; PS, allowed region on the dipeptide map corresponding to $-180^\circ<\phi<0^\circ$.

c) R, distance between C_1 and C_{13}.

$c(PSS)_2$ was shown to be thermodynamically flexible in the sense that cis as well as trans conformations of the six peptide bonds are stable.

With the calculation using the hard-sphere model, the ease of cyclization of linear hexapeptides having different sequences of cis and trans peptide bonds was determined. In natural cyclic peptides the peptide bond sometimes deviates from planarity and the bond lengths and the bond angles are different from those in linear peptides due to the strain. These points were seriously taken into account in the calculation; that is , the geometries of the sarcosine and proline residues were taken so that the allowed region for the peptide map was as wide as possible, and the end-to-end distance was taken as distantly as 4 Å. Furthermore, the degree of atomic overlapping was always checked with the chains which were rejected during the calculation on the basis of the atomic overlap check. This led us to the conclusion that four kinds of C_2-symmetric conformations having different sequences of peptide bonds are allowed for the chains having an end-to-end distance is shorter than 2.5 Å. Some conformers detected in nmr spectra including cis-cis (P_1P_3) conformer were found not to be allowed in the calculation. Cis-trans-cis and cis-cis-cis ($P_1P_2P_3$) conformers produced severe atomic overlapping in the calculation. This disagreement between the calculation and the nmr spectra leads to the following consideration; the C_2-symmetric conformations appearing in the nmr spectra must be C_2-symmetric only on the nmr time scale. Some asymmetric conformations which involve C_2-symmetric sequences of peptide bonds could be averaged out by a rapid rotation around single bonds. The same idea has

been proposed for enniatin(15) and cyclo(Pro-Gly)$_2$(16), which is very interesting from the point of the dynamics of the cyclic peptide backbone.

Cyclo(PSS)$_2$ which consists of N-substituted amino acids, was shown to possess kinetic flexibility as well as thermodynamic flexibility, in spite of the cyclic constraint. The former is related to a rapid rotation around some single bonds and the latter is related to a cis/trans isomerization of six peptide bonds. The investigation of flexibility should be important in determining the conformational adaptability on the host-guest complexation or in analysing the dynamic equilibrium of the complexation.

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Chapter 2

Synthesis and Conformation of Cyclic Pentapeptide Containing a Tryptophan Residue

INTRODUCTION

To clarify the relation between the structure and the function of peptide, a number of cyclic peptides have been synthesized and the conformation/function relationship has been investigated by various spectroscopic methods(1-4). For example, to inquire the information about the action of ionophores, the conformations of model cyclic peptides in solution have been investigated with X-ray diffraction, cd, and nmr spectroscopy(5-13). From these investigations it was found that the conformation of peptide is strongly dependent on the solvent. It was therefore suggested that the structure/function relationship of peptide in lipid membrane should be investigated to elucidate the activity of peptide in biological membrane. Some studies on peptides in liposome and micelles have been carried out by cd and nmr spectroscopy(14,15). However, in these investigations the probe concentrations employed were considerably higher than the real ones, and the structure of membrane might have been perturbed. On the other hand, the fluorescence method is advantageous for low probe concentrations to avoid the above complexities. So, the synthesis of cyclic peptide containing a tryptophan residue as a fluorescent probe was attempted to investigate the

conformation and the interactions of the peptide under different circumstances by fluorescence as well as cd and nmr spectroscopy. In this study, cyclo(Try-Sar-Sar-Lys(DNS)-Pro) was synthesized. This cyclic pentapeptide contains three N-substituted peptide bonds, which make the peptide lipophilic and make the conformation versatile due to the cis/trans isomerization. These properties should be advantageous to form specific and stable complexes with metal ions in organic solvents.

EXPERIMENTAL

Synthesis

The synthetic route of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) is shown in Figure 1. The Try residue was protected by the formyl group to avoid the oxidation during the treatment by acid(16).

Cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro)

Boc-Try(CHO)-Sar-Sar-Lys(Z)-Pro-OH was synthesized by the stepwise elongation in liquid phase. To the dimethylformamide solution of Boc-Try(CHO)-Sar-Sar-Lys(Z)-Pro-OH (2.1g) and HOSu (0.44g), DCCI (0.63g) was added at 0°C. After stirring the solution overnight, the solvent was removed under reduced pressure. To the residue ethyl acetate containing a few drops of acetic acid was added, and the precipitation was filtered off. The filtrate was washed with water and dried over sodium sulfate. After the solvent was evaporated to dryness, anisole (1ml) and TFA (15ml) were added at 0°C. After 30 min the solution was concentrated to a small volume. With the addition of dry ether, white precipitation was

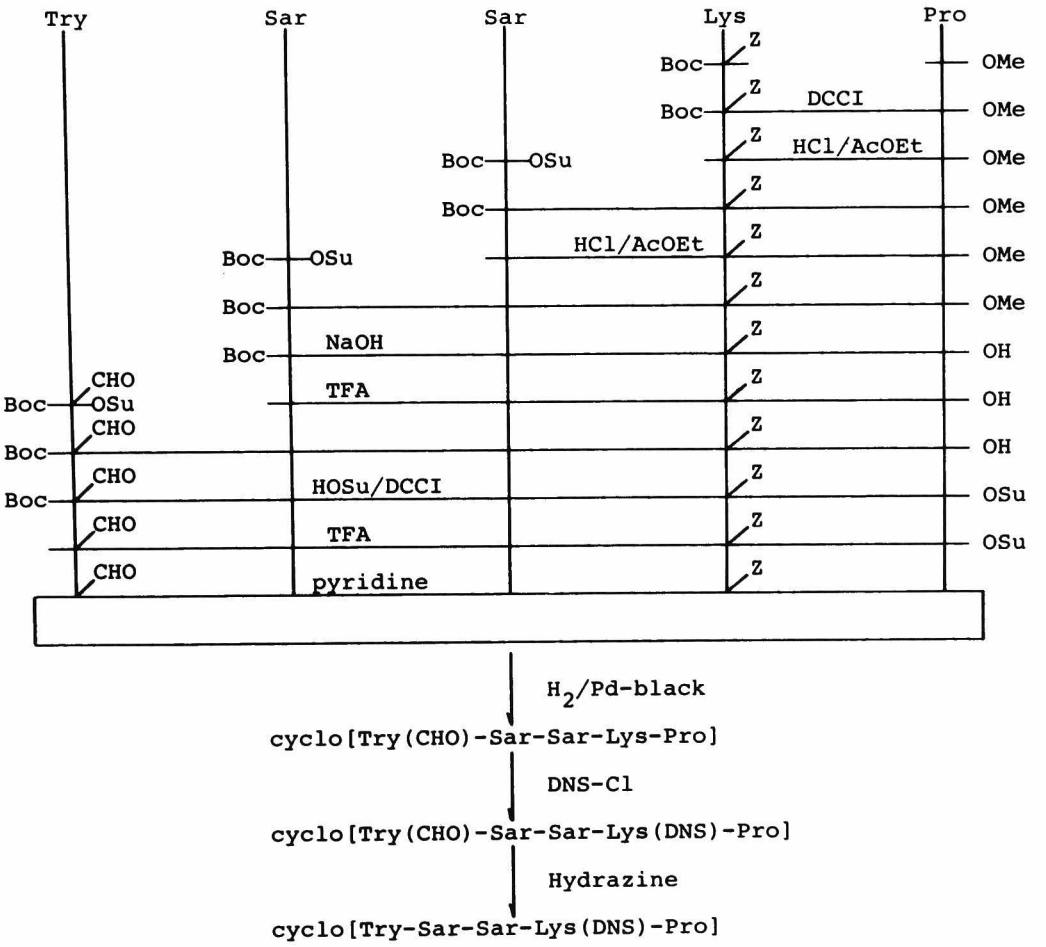

Fig. 1. Synthetic route of cyclic pentapeptide cyclo(Tyr-Sar-Sar-Lys(DNS)-Pro).

collected and dried over NaOH under reduced pressure. The product TFA·Try(CHO)-Sar-Sar-Lys(Z)-Pro-OSu was dissolved in dimethylformamide containing a few drops of acetic acid and the solution was added dropwise to pyridine at 30°C. After the solvent was evaporated off, the residue was dissolved in chloroform, and the solution was purified by gel permeation chromatography using a JASCO MEGAPAK GEL 201 column. Further purification was carried out by a silica gel column using ethyl acetate/methanol (3/1 v/v) as an eluent. The product was dissolved in a small amount of ethyl acetate, and reprecipitated with n-hexane to obtain cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro). Yield from Boc-Try(CHO)-Sar-Sar-Lys(Z)-Pro-OH; 35%. TLC: R_f(I), 0.10; R_f(IV), 0.25; R_f(V), 0.22. ANAL. Calcd. for $C_{37}H_{45}O_8N_7 \cdot 1/2H_2O$; C, 61.31%; H, 6.40%; N, 13.53%. Found: C, 61.44%; H, 6.58%; N, 13.28%.

Cyclo(Try-Sar-Sar-Lys(DNS)-Pro)

Cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) (0.173g) was dissolved in methanol containing acetic acid (17µl), and hydrogenated using Pd/black as a catalyst. The catalyst was filtered off and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform, and triethylamine (81µl) and dansyl chloride (78mg) in chloroform were added. The solution was then concentrated and eluted through a gel permeation chromatography column to obtain cyclo-(Try(CHO)-Sar-Sar-Lys(DNS)-Pro). Cyclo(Try(CHO)-Sar-Sar-Lys(DNS)-Pro) (88mg) was dissolved in a mixture of dimethylformamide (1.4ml) and hydrazine monohydrate (0.135ml), and the solution was left for standing for

48 hours(17). Sufficient water was added to cause precipitation, which was washed with water. It was purified by eluting the solution through a GPC column and finally pure cyclo(Try-Sar-Sar-Lys(DNS)-Pro) was obtained. TLC: R_f(I), 0.03; R_f(IV), 0.28. ANAL. Calcd. for $C_{40}H_{50}O_7N_8S$: C, 61.05%; H, 6.40%; N, 14.24%; S, 4.07%. Found: C, 60.76%; H, 6.61%; N, 14.02%; S, 3.88%.

Cyclo(Try-Sar-Sar-Lys(Z)-Pro) was also obtained from cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) by the treatment with hydrazine.

Measurement

The chemical shifts of ^{13}C nmr spectra were obtained using solvent signals as standard (CD_3OD, 48.0 ppm; CD_3CN, 1.3 ppm; $CDCl_3$, 76.9 ppm).

RESULTS AND DISCUSSION

Conformation of Cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro)

^{13}C nmr spectra of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) in $CDCl_3$, CD_3CN, and CD_3OD are shown in Figure 2. The assignment of each signal in $CDCl_3$ is indicated in the figure, which is based on its chemical shift and the off-resonance ^1H-^{13}C double resonance experiments. Only one signal appears for each carbon atom, indicating that this cyclic pentapeptide takes one conformtion in these solvents on the nmr time scale. Comparison of these spectra suggests that the conformation in these solvents

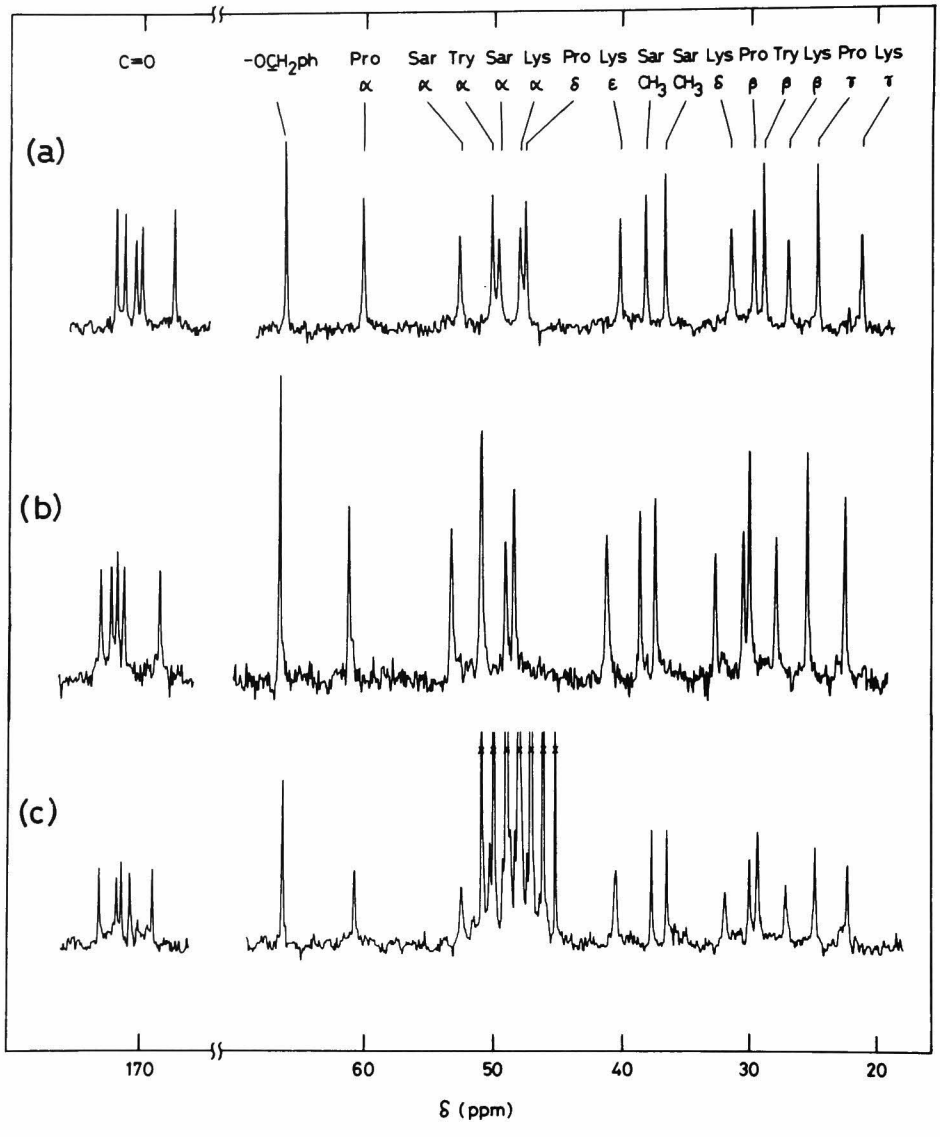

Fig. 2. ^{13}C nmr spectra of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) in (a) $CDCl_3$, (b) CD_3CN, and (c) CD_3OD; x means the signal of solvent.

is the same. On the other hand, in DMSO-d_6 several signals appeared for each carbon atom, indicating that several conformations coexist (data are not shown). These conformers are considered to be the cis/trans isomers with reference to the N-substituted peptide bonds.

In ^1H nmr, the addition of benzene-d_6 to the chloroform solution of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) induced the shifts of sarcosyl N-CH_3 signals as shown in Figure 3. The signal shifted strongly to higher magnetic field should be ascribed to the N-CH_3 protons adjacent to a trans peptide bond and the signal shifted slightly to higher magnetic field to those to a cis peptide bond(18). This assignment allows the signals appearing at 36.6 and 38.1 ppm in ^{13}C nmr spectrum (Figure 2(a)) to be assigned to N-CH_3 carbons adjacent to a trans and a cis peptide bond, respectively, by the selective irradiation of each N-CH_3 protons in ^{13}C nmr spectrum.

Signals of Pro-C^{β} and Pro-C^{γ} are found in the usual region for trans peptide bond(2). Therefore, the peptide bonds in this cyclic peptide are trans with one cis of the Sar peptide bond.

The temperature dependences of the chemical shifts of NH signals in CD_3CN were found to be 7.3×10^{-3} ppm$\cdot$deg^{-1} for urethane amide proton, and 3.0×10^{-3} and 3.1×10^{-3} ppm$\cdot$deg^{-1} for amide protons. Ir spectrum of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) in chloroform showed a strong absorption at 3294 cm^{-1}, and the extinction coefficient was independent of the concentration. Therefore, the amide protons of the cyclic skeleton should form intramolecular hydrogen bondings (19,20). As proton acceptors for the hydrogen-bonded amide protons, five carbonyl groups of the cyclic skeleton and a urethane

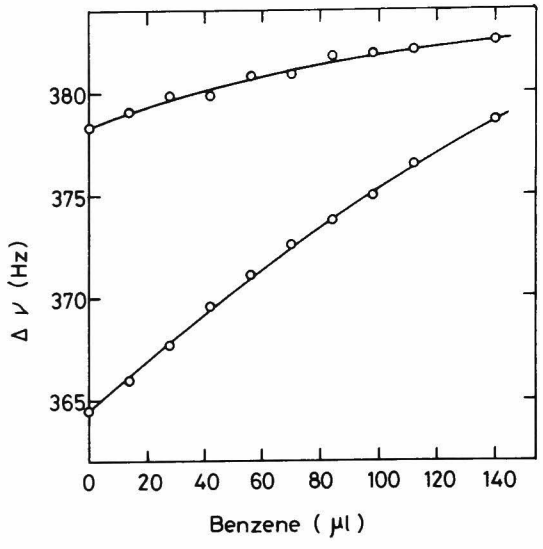

Fig. 3. Change of the chemical shifts of N-CH_3 signals of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) with the addition of benzene-d_6 in $CDCl_3$. Δυ represents the difference of the frequencies of N-CH_3 signal from phenyl signal as standard.

group of the side chain are considered. Inspection of the Corey-Pauling-Koltun molecular model revealed that the latter carbonyl group can form an intramolecular hydrogen bond with Lys-NH, but not with Try-NH. β-Turn or γ-turn is often found in cyclic peptides containing intramolecular hydrogen bonding. If a γ-turn (1←3 intramolecular hydrogen bonding) occurs in the present case, the structure shown in Figure 4(a) or (b) might be drawn. In either case a Pro residue is involved in the γ-turn. For this structure the signal of Pro-C^{β} in ^{13}C nmr spectrum has been reported to shift to higher magnetic field(2). This was not observed in the present case, so that the structures of Figures 4(a) and (b) are not likely. If a β-turn (1←4 intramolecular hydrogen bonding) occurs in the present case, the structure shown in Figure 4 (c) or (d) might be considered. The conformation of Figure 4 (c) contains a cis peptide bond in the β-turn. If Try-NH forms an intramolecular hydrogen bonding at the same time, it leads to the formation of the same γ-turn as shown in Figure 4 (b). So, the structure of Figure 4 (c) is also unlikely. In the structure shown in Figure 4 (d) the Pro residue takes the 3rd position in the β-turn, which has been seldom found in nature. Since the structure of Figure 4 (d)-2 is far less sterically hindered than that of (d)-1, the former is the most plausible structure of all possibilities. This conformation is characterized as follows, i) it contains a β-turn in which Pro residue takes the 3rd position, ii) intramolecular hydrogen bonding is formed between the carbonyl group of the side chain and the NH of the cyclic skeleton (the same is found in ferrichrome(21)), and iii) one cis peptide bond is contained in the ring structure (this is observed in cyclo(Ala-Pro-Gly-D-Phe-

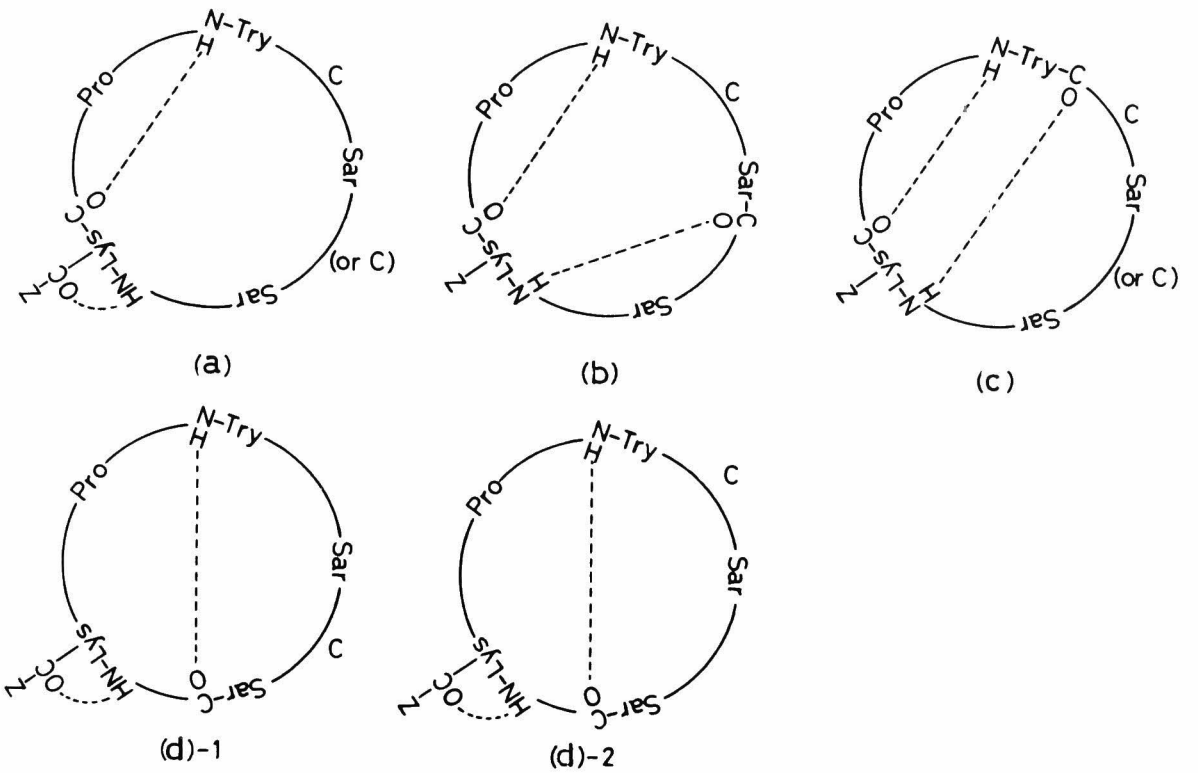

Fig. 4. Possible conformations of cyclo(Try(CHO)-Sar-Sar-Lys(Z)-Pro) with two amide protons forming intramolecular hydrogen bonds (dotted line) of β-turn type, γ-turn type or with urethane group. C represents a cis peptide bond.

Pro) (14)). The above discussion assumes the formation of 1←3 or 1←4 intramolecular hydrogen bonding. The occurrence of other structures than those cannot be excluded.

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Chapter 3

Synthesis and Conformation of Cyclic Octapeptides, Cyclo(Phe-Pro)$_4$, Cyclo(Leu-Pro)$_4$, and Cyclo(Lys(Z)-Pro)$_4$

INTRODUCTION

Cyclic peptides having various sizes of ring skeleton have been synthesized, and their conformations and interactions with ions have been investigated(1). In the case of bis-cyclic dipeptides,in which two cyclic dipeptides are connected, 2:1 peptide/metal ion complex was formed and the ion was captured by four carbonyl groups(2). The cyclic pentapeptide cyclo(Gly-Pro-Gly-D-Ala-Pro) has been reported to form a 1:1 complex with its three or even two carbonyl groups(3). Cyclic hexapeptide cyclo(Gly-Pro)$_3$ (4) and cyclic octapeptide cyclo(Gly-Pro)$_4$ (5) have been shown to form a 1:1 complex with metal ion due to intramolecular cooperation of three and four carbonyl groups, respectively. The naturally-occurring cyclic decapeptide antamanide forms a complex with Li^+ with its four carbonyl groups(6).

On the other hand, the ability of complex formation of cyclic decapeptide cyclo(Gly-Pro)$_5$ has been reported to be inferior to that of cyclo(Gly-Pro)$_4$ (5). These results indicate that if three of four carbonyl groups cooperatively coordinate to an ion, stable complexes are formed.

Cyclic peptides smaller than octapeptide tend to form a metal ion complex in which the metal ion is

bound on the plane of the cyclic skeleton. Cyclic peptides larger than decapeptide usually take a folded structure because of their size,and form an inclusion-type complex with metal ion as seen with antamanide/Na^+ complex. In a series of cyclic depsipeptides, an inclusion-type complex has been observed with a cyclic hexadepsipeptide enniatin(7).

On the basis of the above information, all-L cyclic octapeptides seem to be an efficient ion carrier, and their synthesis and the investigations on the conformational properties are desirable. In this chapter, a series of cyclic octapeptides cyclo$(X\text{-}Y)_4$ in which Y represents Pro, and X represents Phe, Leu, or Lys(Z) were synthesized, and their conformational properties were compared. Pro residue enhances the lipophilicity of peptide as well as allowing a cis/trans isomerization of peptide bond. The latter character increases the number of available conformations of peptide, which has been considered to be a favorable situation for complex formation.

EXPERIMENTAL

Synthesis of Cyclic Octapeptides

Synthetic routes to cyclic octapeptides are shown in Figures 1, 2, and 3. Condensation reactions were carried out using either DCCI or DCCI/HOBt mixture, or by the active ester method. Boc group was removed by the treatment with HCl/AcOEt or TFA, and an ester group was removed by hydrogenation with Pd/black or saponification. Linear octapeptides were obtained by the fragment

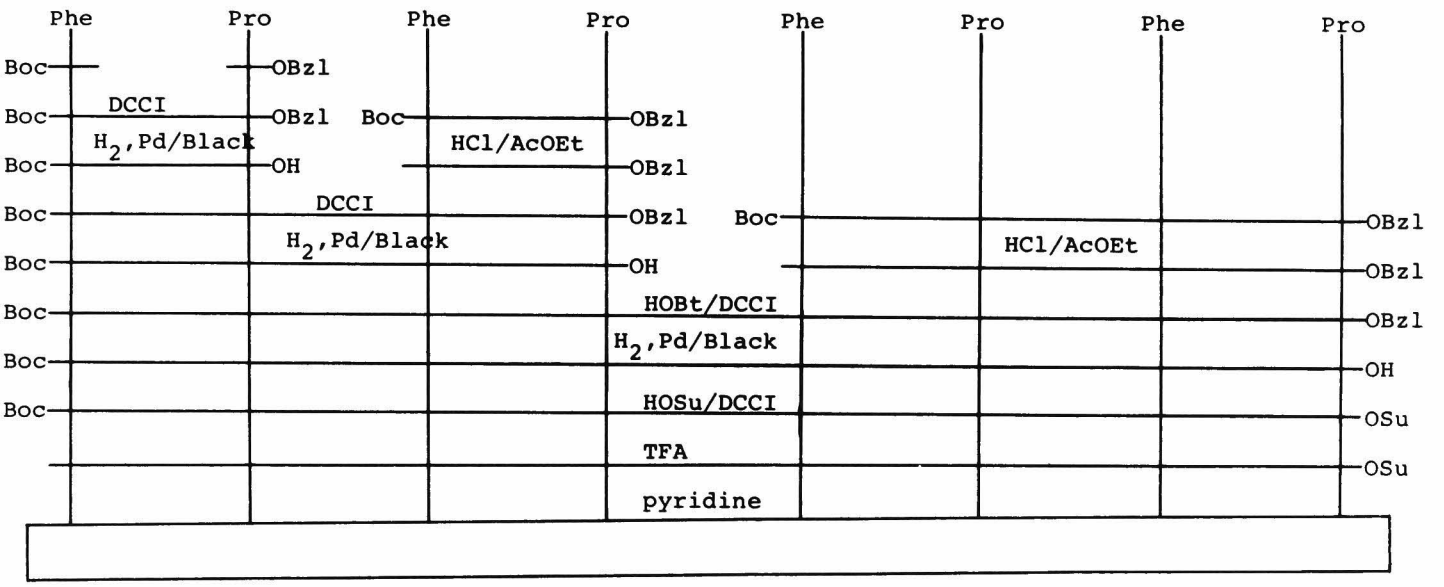

Fig. 1. Synthetic route of cyclo(Phe-Pro)$_4$.

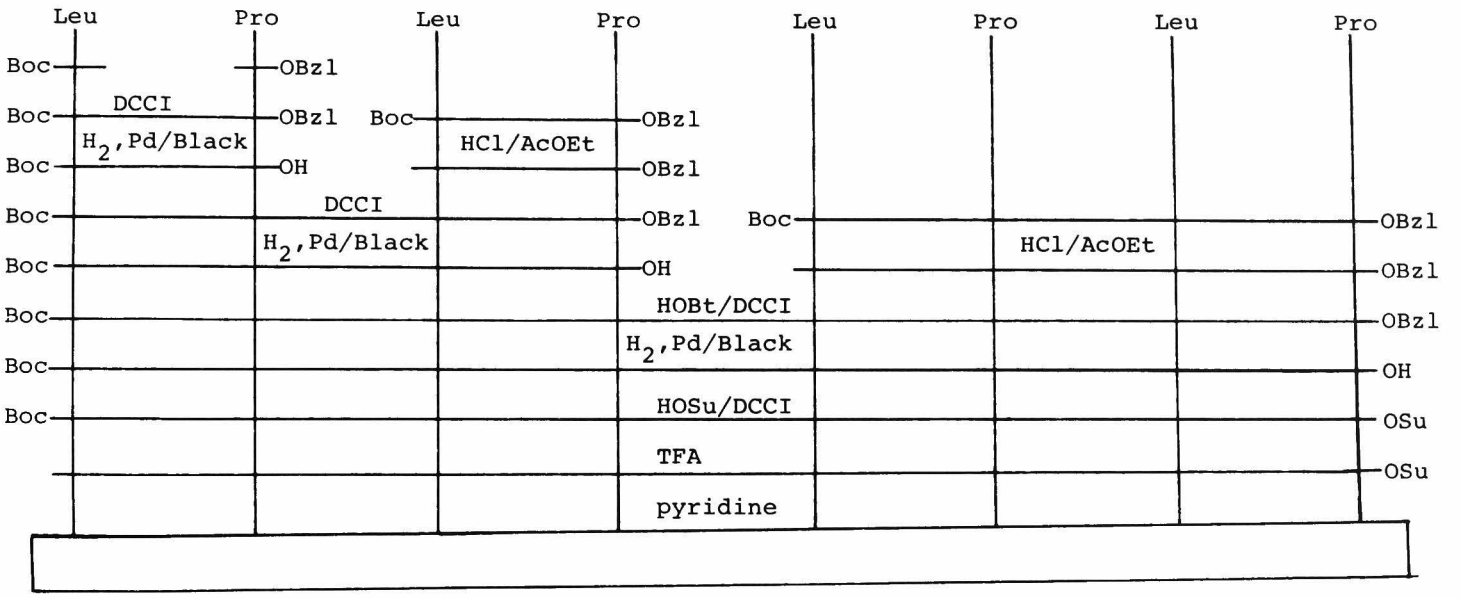

Fig. 2. Synthetic route of cyclo(Leu-Pro)$_4$.

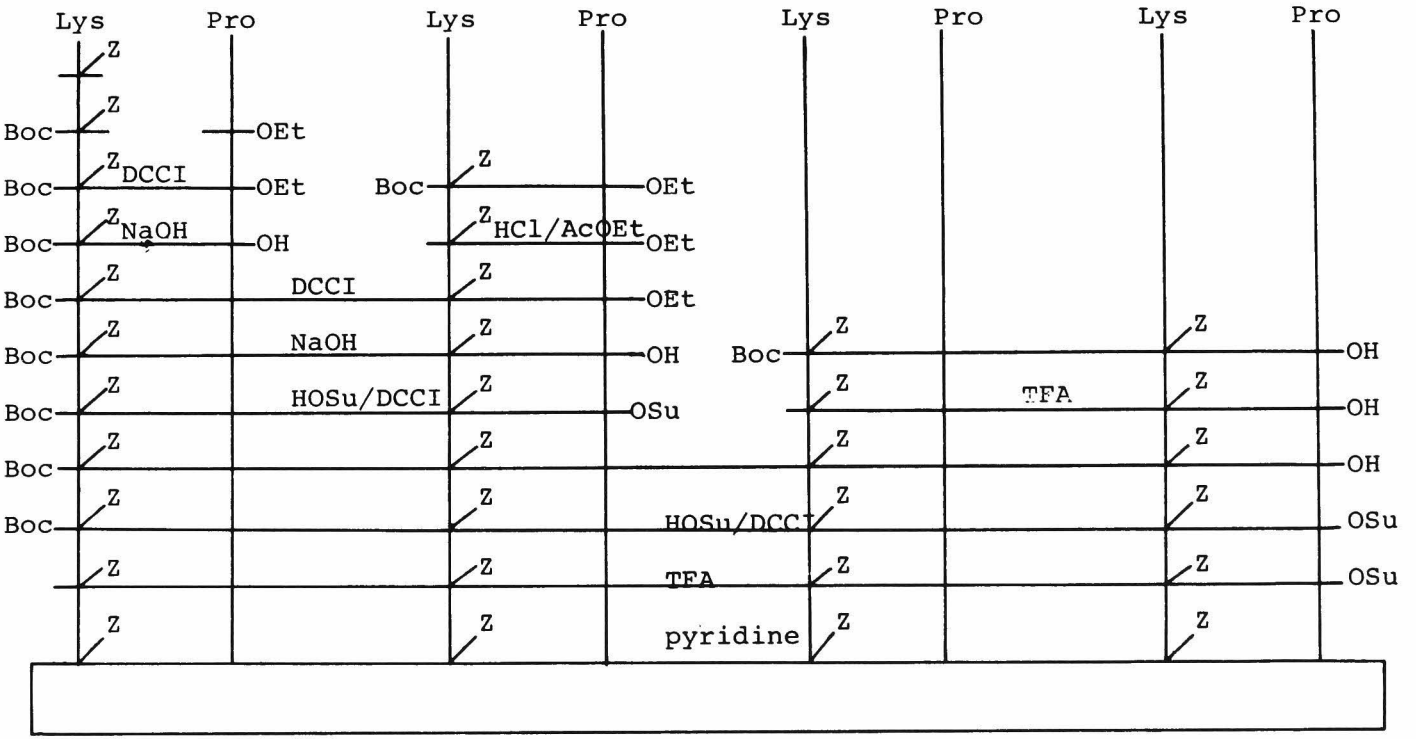

Fig. 3. Synthetic route of cyclo(Lys(Z)-Pro)$_4$.

condensations, and then they were cyclized to produce cyclic octapeptides.

Cyclo(Phe-Pro)$_4$

Boc-(Phe-Pro)$_4$-OBzl was subjected to a catalytic hydrogenation with Pd/black in ethyl acetate to obtain Boc-(Phe-Pro)$_4$-OH. It was recrystallized from ethyl acetate/n-hexane. TLC: R_f(III), 0.08. To a dimethylformamide solution of Boc-(Phe-Pro)$_4$-OH (0.584g) and HOSu (0.092g), DCCI (0.165g) was added at 0°C. After stirring overnight, the solution was concentrated in vacuum. The residue was dissolved in ethyl acetate, a few drops of acetic acid were added, and the solution was filtered. The filtrate was washed with 4% $NaHCO_3$ aqueous solution and water and dried over Na_2SO_4. The solvent was evaporated to dryness, and TFA (7ml) was added to the residue at 0°C. After 30 min the volatile matter was evaporated, and dry ether was added to yield a white precipitate. After filtration, the white mass was dried over NaOH for 3 hours. The solid was dissolved in dimethylformamide (15ml) containing a few drops of acetic acid, and the solution was added in dropwise into pyridine (250ml) at 30°C. After stirring overnight, the solvent was distilled off. The residue was dissolved in CH_3OH/H_2O (4/1) mixture, and the solution was eluted through the columns of Dowex 1 and Dowex 50. The eluted solution was condensed in vacuum to obtain a white solid. This was extracted with ethyl acetate, and after concentration, petroleum ether was added to precipitate a white solid. This was recrystallized from ethyl acetate/n-hexane mixture. Yield from Boc-(Phe-Pro)$_4$-OH, 42%. TLC: R_f(I), 0.36;

R_f(II), 0.81; R_f(III), 0.12. ANAL. Calcd. for $C_{56}H_{64}O_8N_8 \cdot 3H_2O$; C, 65.23%;H, 6.84%; N, 10.87%. Found: C, 65.22%; H, 6.64%; N, 10.83%. FDMS: m/e; 976.

Cyclo(Leu-Pro)$_4$

Boc-(Leu-Pro)$_4$-OBzl which was synthesized by fragment condensation was purified by GPC with JASCO MEGAPAK GEL 201 using $CHCl_3$ as an eluent. Boc-(Leu-Pro)$_4$-OBzl was subjected to a catalytic hydrogenation with Pd/black in t-butanol/CH_2Cl_2 mixture to obtain Boc-(Leu-Pro)$_4$-OH. To a dimethylformamide solution of Boc-(Leu-Pro)$_4$-OH (1.955g) and HOSu (0.352g), DCCI (0.484g) was added at 0°C. After stirring overnight, the solution was concentrated in vacuum, and the residue was dissolved in ethyl acetate. After the precipitate was filtered off, the filtrate was eluted through the silica gel column (2.1cm x 6.5cm) using ethyl acetate/CH_3OH mixture as an eluent. Anisole (1ml) and then TFA (12ml) were added to Boc-(Leu-Pro)$_4$-OSu at 0°C. After 30 min the volatile matter was evaporated, and a dry ether/petroleum ether mixture was added to yield a white solid, which was recovered by filtration and dried over NaOH. It was dissolved in dimethylformamide (55ml) containing a few drops of acetic acid and the solution was added dropwise to pyridine (800ml) at 30°C. The solvent was condensed in vacuum and the residue was purified by ion exchange using the same procedure as described for cyclo(Phe-Pro)$_4$ and a GPC column.

The reaction product was dissolved in a small amount of ethyl acetate and ether was added to precipitate cyclo(Leu-Pro)$_4$. Yield from Boc-(Leu-Pro)$_4$-OH; 38%. TLC: R_f(I), 0.28; R_f(II), 0.67; R_f(III), 0.23. ANAL.

Calcd. for $C_{44}H_{72}O_8N_8 \cdot H_2O$: C, 61.51%; H, 8.68%; N, 13.04%. Found: C, 61.62%; H, 8.62%; N, 12.93%.

Cyclo(Lys(Z)-Pro)$_4$

Boc-(Lys(Z)-Pro)$_4$-OH was purified by reversephase high-pressure liquid chromatography on a SIL-ODS/C_{18} column with CH_3OH/H_2O (4/1) mixture as a mobile phase. To a dimethylformamide solution of Boc-(Lys(Z)-Pro)$_4$-OH (1.39g) and HOSu (0.154g), DCCI (0.239g) was added at 0°C, and the solution was stirred overnight. This was purified by the same procedure as described for Boc-(Phe-Pro)$_4$-OSu to obtain Boc-(Lys(Z)-Pro)$_4$-OSu. Anisole (0.75ml) and then TFA were added to the product at 0°C. After 30 min the volatile matter was evaporated. Dry ether was added to the residue to yield a white precipitate. Supernatant was decanted and the white mass was dried over KOH. It was dissolved in dimethylformamide (25ml) containing a few drops of acetic acid, and the solution was added dropwise to pyridine (500ml) at 30°C. The solution was concentrated in vacuum, and the residue was purified by ion exchange in the same procedure as employed for cyclo(Phe-Pro)$_4$ and a SIL-ODS/C_{18} column with CH_3OH/H_2O (4/1) mixture as a mobile phase. The eluent was evaporated, and the residue was dissolved in a small amount of ethyl acetate. Ether was added to precipitate cyclo(Lys(Z)-Pro)$_4$. Yield from Boc-(Lys(Z)-Pro)$_4$-OH is 33%. TLC: R_f(I), 0.42; R_f(II), 0.84; R_f(III), 0.20. ANAL. Calcd. for $C_{76}H_{100}O_{16}N_{12} \cdot 3/2\ H_2O$: C, 62.32%; H, 7.09%; N, 11.48%. Found: C, 62.41%; H, 7.05%; N, 11.45%. FDMS: m/e, 1436.

^{13}C Nmr Spectra

The peptide concentration in ^{13}C nmr measurements was $2 \times 10^{-2} \sim 8 \times 10^{-2}$ M.

^{13}C spin-lattice relaxation times (T_1) were acquired using a 180°-τ-90° pulse sequence, and 3 ∿ 3.5 sec interval was allowed between each pulse sequence.

RESULTS AND DISCUSSION

Conformation of Cyclic Octapeptides

^{13}C nmr spectra were measured in various solvents to obtain information about the conformations in solution.

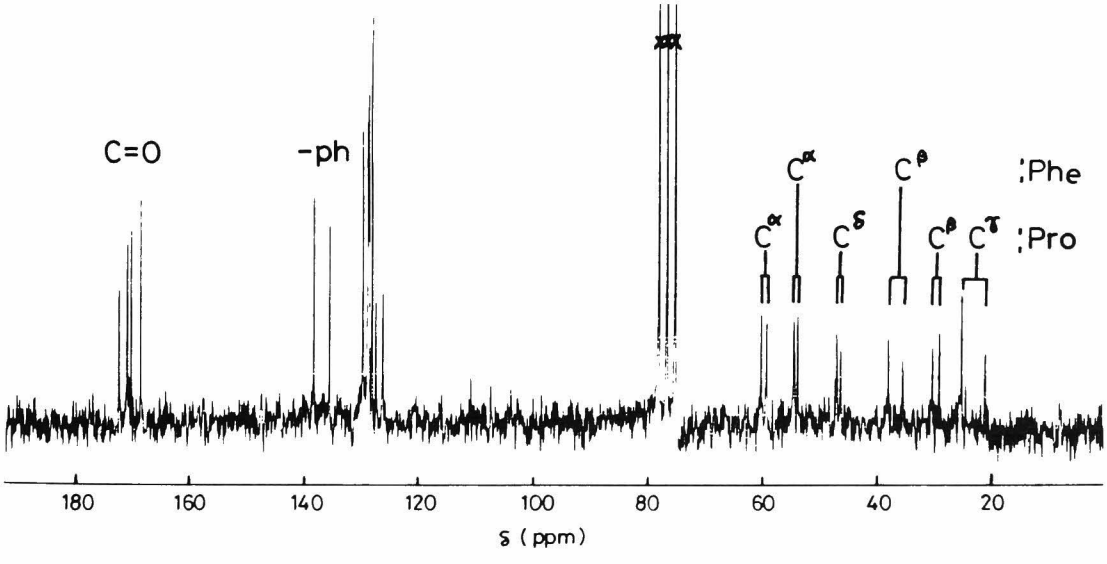

Fig. 4. ^{13}C nmr spectrum of cyclo(Phe-Pro)$_4$ in $CDCl_3$.

All cyclic octapeptides investigated here contain Pro residues which allow cis as well as trans peptide bonds, and this rate of isomerization is slow on nmr time scale so that signals arising from each conformational state can be distinguished. Thus a variety of conformations detected by nmr spectra are the isomers with respect to peptide bonds. Figure 4 shows ^{13}C nmr spectrum of cyclo(Phe-Pro)$_4$ in $CDCl_3$. The assignment of signals is based on their chemical shifts(8). The appearance of two signals for each carbon atom of Pro and Phe residues of cyclo(Phe-Pro)$_4$ indicates that it takes up a C_2-symmetric conformation in this solvent. The signals of C^{β} and C^{γ} carbon of Pro residues were located at 30.52, 29.44 and 25.43, 21.26 ppm, respectively. Correlation between the chemical shifts of C^{β} and C^{γ} atoms of Pro residues and the cis or trans form of the X-Pro bond has been reported(9,10). Accordingly signals at 29.44 and 25.43 ppm and those at 30.52 and 21.26 ppm were ascribed to alkyl carbon atoms adjacent to trans and cis peptide bonds, respectively.

^{13}C nmr spectra of cyclo(Phe-Pro)$_4$ in DMSO-d_6 and CD_3OD/D_2O (95/5) mixture were similar to that in $CDCl_3$. The conformation in DMSO-d_6 was proven to be the same as that in $CDCl_3$, because the successive change of $CDCl_3$/DMSO-d_6 compositions did not make ^1H nmr signals newly appearing or disappearing and did not alter $J_{HN-C^{\alpha}H}$. Therefore, cyclo(Phe-Pro)$_4$ has a C_2-symmetric conformation containing two cis peptide bonds, which is independent of the nature of solvent.

^{13}C nmr spectra of cyclo(Leu-Pro)$_4$ in $CDCl_3$, CD_3CN, DMSO-d_6, and CH_3OD/D_2O (95/5) mixture are shown in Figure 5. The spectral pattern is different according to the nature of solvent, which is different from the

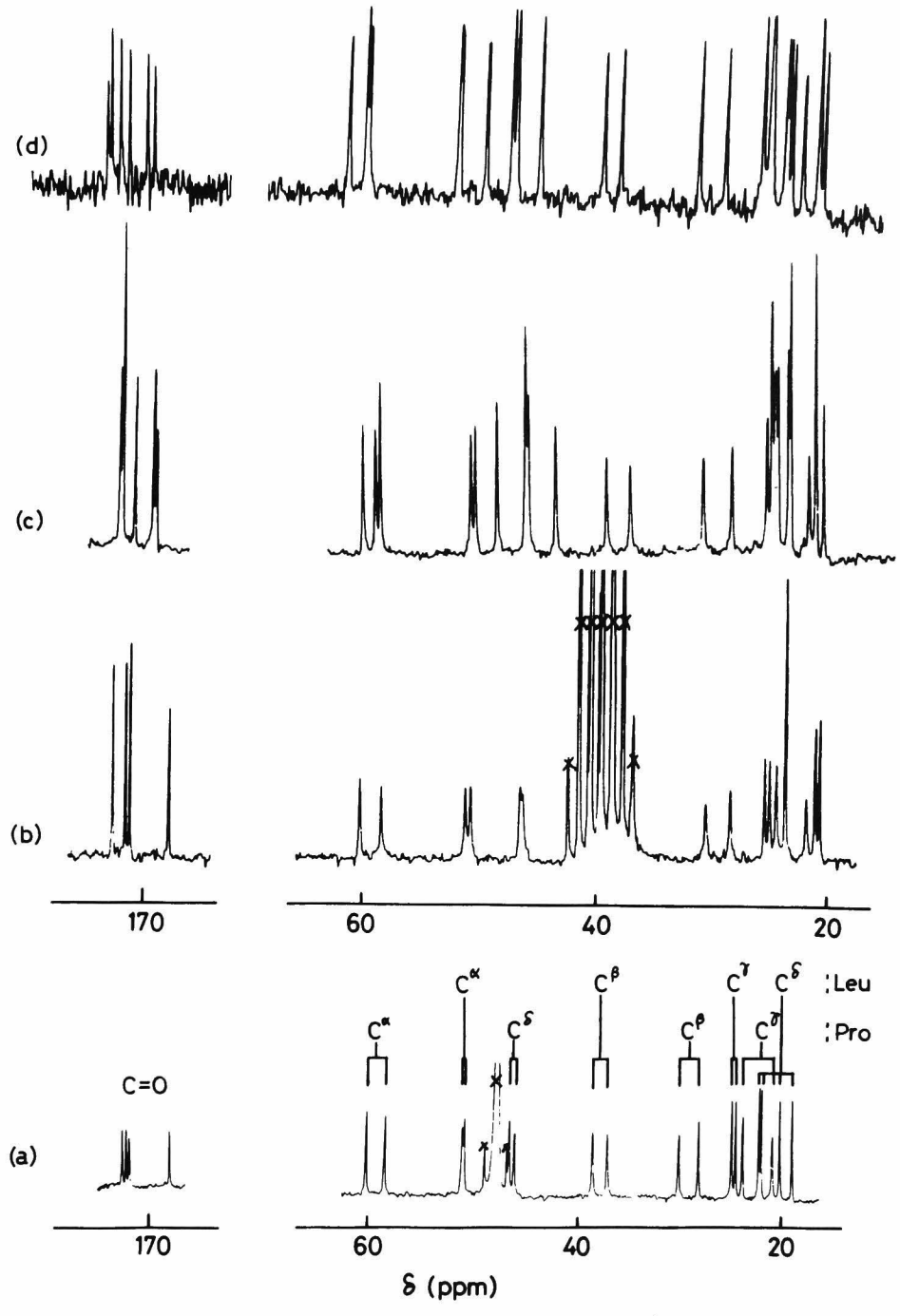

Fig. 5. ^{13}C nmr spectra of cyclo(Leu-Pro)$_4$ (a) in 95% CH_3OD, (b) in DMSO-d_6, (c) in $CDCl_3$, (d) in CD_3CN.

case of cyclo(Phe-Pro)$_4$. In $CDCl_3$ and CD_3CN, three signals appeared for each carbon atom of Leu and Pro residues. This fact indicates that one C_2- and one C_4-symmetric conformers coexist. On the other hand, in DMSO-d_6 and CH_3OD/D_2O (95/5) mixture, two signals appeared for each carbon atom of Leu and Pro residues. Therefore in these solvents, only one C_2-symmetric conformer exists. In this conformer the peptide bonds of Pro residues are cis and trans, because two kinds of signals were observed for C^{β}. Therefore, this conformation seems to be the same as the C_2-symmetric conformation of cyclo(Phe-Pro)$_4$ which contains two cis peptide bonds.

In addition to this C_2-symmetric conformation, C_4-symmetric conformation appeared in CD_3CN and $CDCl_3$. No signal ascribable to C^{β} of Pro residue, however, appeared around 30 ppm region. On the basis of the Ba^{2+} ion addition to cyclo(Leu-Pro)$_4$ in CD_3CN, a signal at 26.66 ppm was assigned to the C^{β} of Pro residue in this C_4-symmetric conformer(11). This unusual shift of Pro-C^{β} signal to a high magnetic field has been observed with cyclo(Pro-Gly)$_3$, which has been reported to take up a γ-turn conformation(10). Therefore, this C_4-symmetric conformation of cyclo(Leu-Pro)$_4$ is likely to contain four γ-turns and all trans peptide bonds.

The conformational distribution of cyclo(Leu-Pro)$_4$ in $CDCl_3$ is similar to that in CD_3CN. A different conformational distribution is found in CH_3OD/D_2O (95/5) mixture which is the same as that in DMSO-d_6. This fact indicates that the stability of each conformation does not depend on the polarity of solvent but on the energy of solvation involving hydrogen bonding, etc.

^{13}C nmr spectra of cyclo(Lys(Z)-Pro)$_4$ in $CDCl_3$ and DMSO-d_6 are shown in Figure 6. In DMSO-d_6 two signals were observed for each of Lys(Z)-C^{α} and Pro-C^{α} and C^{δ}, so that cyclo(Lys(Z)-Pro)$_4$ takes up a C_2-symmetric conformation containing two cis peptide bonds. The ^{13}C nmr spectrum in CD_3OD is similar to that in DMSO-d_6, and the signals appearing in 21-26 ppm region can be assigned as follows:25.09 ppm for trans-Pro-C^{γ};23.79 and 22.43 ppm for Lys(Z)-C^{γ}; 21.62 ppm for cis-Pro-C^{γ}. This assignment is consistent with the C_2-symmetric conformation containing two cis peptide bonds. The signal appearing at 21.62 ppm was assigned to cis-Pro-C^{γ} because of its short T_1 value. The short T_1 value represents the rigidity of pyrrolidine ring connected to a cis peptide bond(18). The mobility of pyrrolidine ring will be discussed in details later.

All signals in ^{13}C nmr spectrum of cyclo(Lys(Z)-Pro)$_4$ in $CDCl_3$ were not well resolved. Many resonance signals arising from the mixture of several kinds of conformations seem to have overlapped. The available numbers of isomers of these cyclic octapeptides with respect to peptide bonds are six as following; all trans and C_4, four cis and C_4, two cis and C_2 or asymmetric, and one or three cis and asymmetric. Cyclo(Lys(Z)-Pro)$_4$ in $CDCl_3$ should be a mixture of several conformations of the above.

It was shown that cyclo(Phe-Pro)$_4$, cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ take a C_2-symmetric conformation containing two cis peptide bonds in DMSO-d_6 and CD_3OD. The details of this conformation will be described in the next paragraph. On the other hand, available conformations in $CDCl_3$ were different according to the nature of cyclic cotapeptides. Numbers of possible isomers

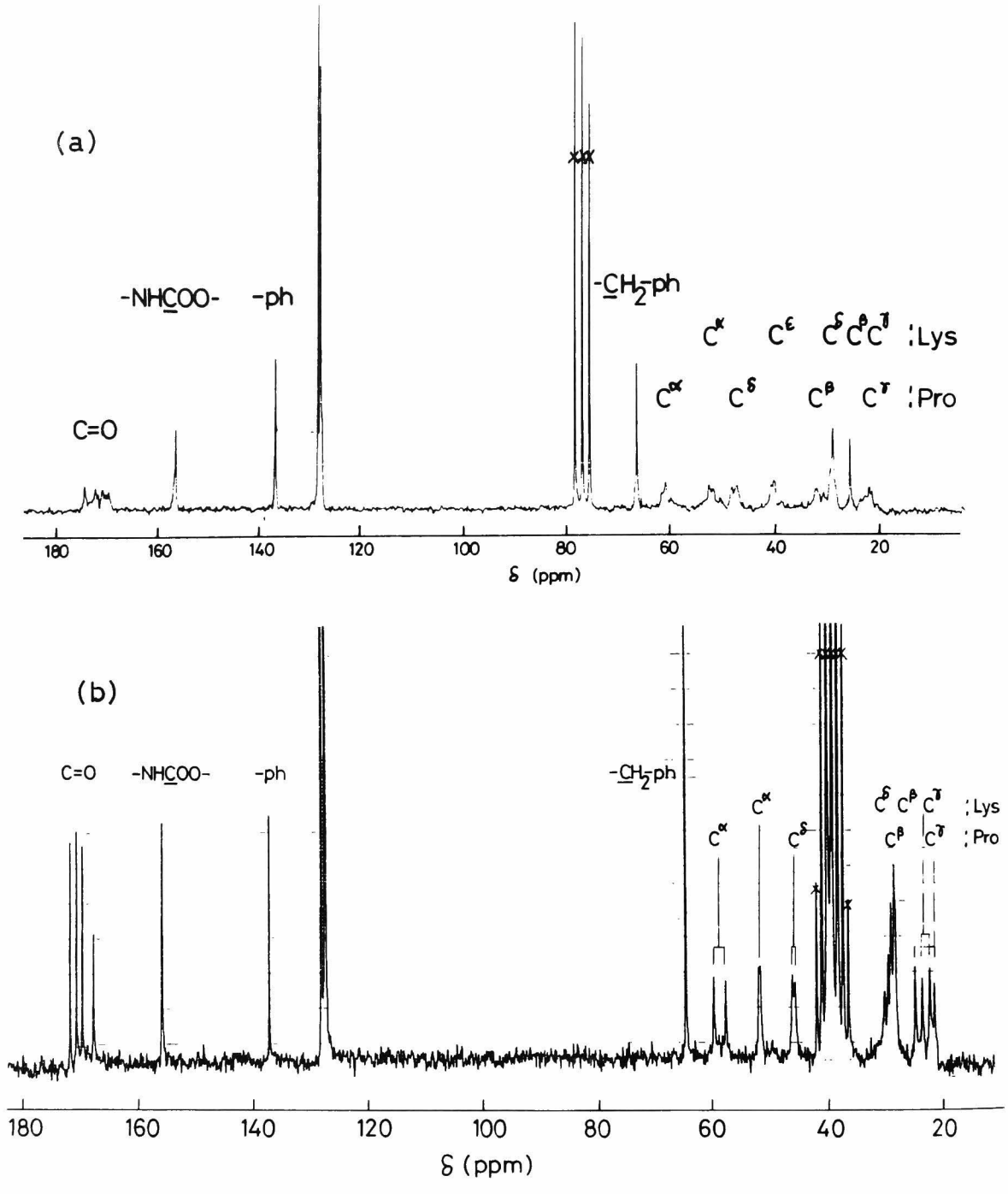

Fig. 6. ^{13}C nmr spectra of cyclo(Lys(Z)-Pro)$_4$ (a) in $CDCl_3$, (b) in DMSO-d_6.

with respect to peptide bonds decreased in the order: cyclo(Lys(Z)-Pro)$_4$ > cyclo(Leu-Pro)$_4$ > cyclo(Phe-Pro)$_4$. The allowed region of ϕ and ψ of oligopeptides are generally influenced by the steric repulsion between side chains of amino acid residues. A similar situation will be met in cyclic peptides, too. In the series of cyclic octapeptides investigated here, the steric repulsion between C^{δ} of Pro and C^{β} and C^{γ} of the other residue seems to be important to determine the main chain conformation. The order of conformational availability in $CDCl_3$ mentioned above might be explained as following; the steric repulsion between side chains decreases in the order Phe > Leu > Lys(Z), and the less the repulsion is, the more the available conformations of ring skeleton increase.

Conformation of Cyclo(Phe-Pro)$_4$

Cyclo(Phe-Pro)$_4$ was shown to take up a C_2-symmetric conformation containing two cis peptide bonds in $CDCl_3$, DMSO-d_6, and CD_3OD. This conformation was further investigated in relation to the intramolecular hydrogen bonding.

The temperature dependences of chemical shifts of two amide protons in DMSO-d_6 are shown in Figure 7. The temperature coefficients of signals at low and high magnetic field were 0.0019 and 0.0057 ppm/^{o}C, respectively, which may indicate that the former forms an intramolecular hydrogen bonding(12). This conclusion was supported by the NH stretching region of infrared spectrum in $CHCl_3$ (Figure 8). Two absorptions at 3425 and 3265 cm^{-1} are assigned to free and hydrogen-bonded NH, respectively

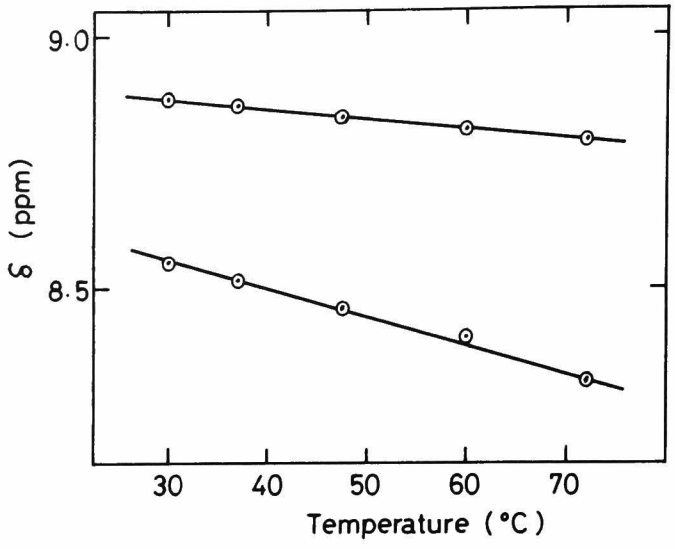

Fig. 7. The temperature dependences of NH resonance signals of cyclo(Phe-Pro)$_4$ in DMSO-d$_6$.

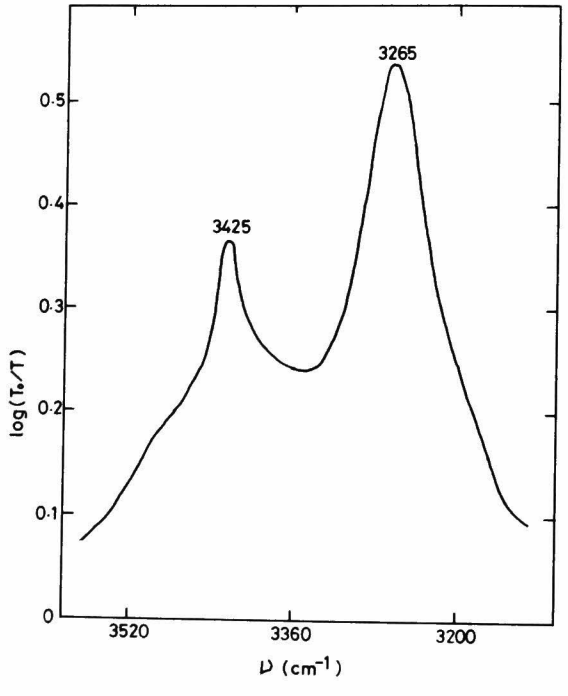

Fig. 8. Ir spectrum of NH stretching region of cyclo(Phe-Pro)$_4$ in CHCl$_3$.

(13). The latter was proven to be intramolecularly hydrogen-bonded, because the extinction coefficient was independent of the concentration.

The ^{1}H-^{13}C double resonance experiment was carried out to elucidate the assignment of carbonyl carbons(14). Figure 9 shows the carbonyl carbon resonance region of ^{13}C nmr spectra in $CDCl_3$. Intensities of two signals at higher magnetic field were enhanced by the selective irradiation of Pro-$C^{\delta}H$. Therefore, these signals are assigned to the carbonyl carbons of Phe residues. Figure 10 shows the solvent dependence of chemical shifts of carbonyl carbons in ^{13}C nmr spectra. By adding D_2O to DMSO-d_6 solution the signal of carbonyl carbon of Pro residue located at the lowest magnetic field is shifted to a less extent than other signals. Therefore, this carbonyl group must participate in an intramolecular hydrogen bonding(15).

Two types of conformations of cyclo(Phe-Pro)$_4$, as shown in Figures 11 (a) and (b), are consistent of the experimental findings. In either case two sets of β-turns (1 ← 4 intramolecular hydrogen bonding) are involved and Pro residues occupy the position 3. Each conformation was examined by constructing a Corey-Pauling-Koltun (CPK) model, and the values of $J_{HN\text{-}C^{\alpha}H}$ were calculated and compared with the experimental values. The observed coupling constants were 8.3 Hz for the hydrogen-bonded NH (less dependent on temperature) and less than 3 Hz for the solvent-exposed NH (highly dependent on temperature). These values agreed very well with those calculated for the conformation of Figure 11 (a), but not with those for Figure 11 (b). In the latter case, the calculated values for the different types of NHs were very small. Therefore, the conformation shown in Figure 11

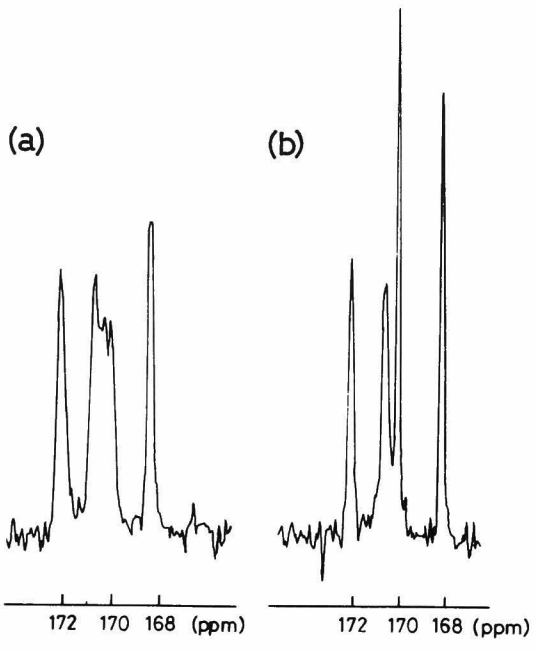

Fig. 9. Carbonyl resonances in the ^{13}C nmr spectra of cyclo(Phe-Pro)$_4$ in $CDCl_3$ (a) without proton irradiation, (b) selective double resonance irradiation at the δ protons of proline residues.

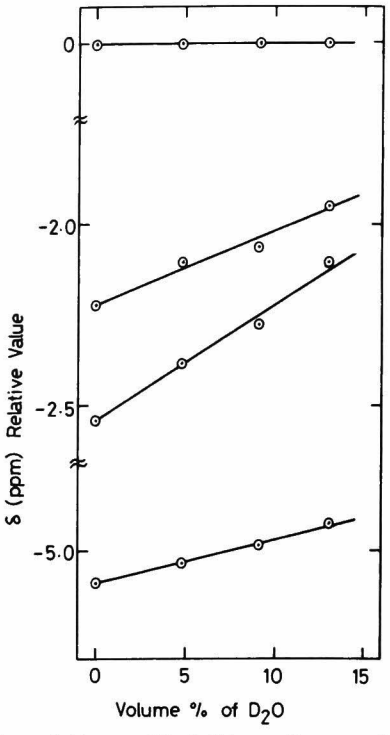

Fig.10. Shift of carbonyl resonances of cyclo(Phe-Pro)$_4$, with the change of solvent composition of DMSO-d_6/D_2O mixture, the lowest field signal being as the reference.

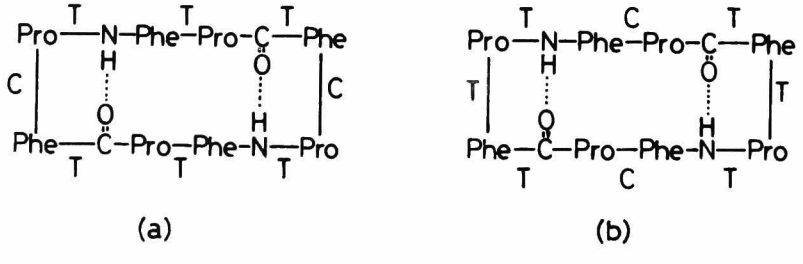

Fig. 11. Possible conformations of cyclo(Phe-Pro)$_4$. T and C represent trans and cis peptide bond, respectively. Dotted lines mean hydrogen bonds.

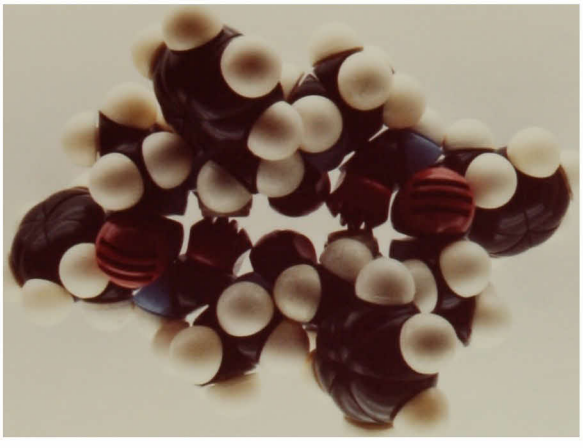

Fig. 12. Proposed conformation of cyclo(Phe-Pro)$_4$ in a free state by CPK model.

(a) is more plausible than that in Figure 11 (b). The β-turn structure in which a Pro residue takes the position 3 in L-L sequence and a cis peptide bond is involved is very unique for synthetic peptides. A few examples of such kind have been found in nature(16). The Corey-Pauling-Koltun model is shown in Figure 12.

^{13}C Spin-lattice Relaxation Times and Conformational Flexibility of Cyclic Octapeptides

^{13}C spin-lattice relaxation times of cyclic octapeptides were measured to investigate the dynamic flexibility (mobilities on the basis of rotations around ϕ, ψ) of the cyclic octapeptides. Table I shows NT_1 of

TABLE I

Spin-Lattice Relaxation Times NT_1 (sec)#) of the Carbon Atoms in Cyclic Peptides; (a) 63 mg/ml in CH_3OD, (b) 122 mg/ml in CH_3OD, (c) 24 mg/ml in CD_3OD, (d) 57 mg/ml in CH_3OD, (e) 80 mg/ml in CH_3OD.

	cyclo(Phe-Pro)$_4$			m.w. 977.176	
	Pro			Phe	
	C^α	C^β	C^γ	C^α	C^β
(a)	0.26	0.28*	0.30*	0.21	0.20
	0.31	0.46	0.72		0.28
(b)	0.20	0.22*	0.24*	0.19	0.20
	0.21	0.36	0.54		0.24

	cyclo(Leu-Pro)$_4$			m.w. 841.107				
	Pro			Leu				
	C^α	C^β	C^γ	C^α	C^β	C^γ	C^δ	$C^{\delta'}$
(c)	0.46	0.62*	0.56*	0.37	0.50	0.63	1.80	1.86
	0.49	0.74	0.96	0.46	0.60	0.68	2.34	2.37
(d)	0.43	0.54*	0.54*	0.38	0.42	0.61	1.83	1.80
	0.45	0.76	1.04	0.42	0.52	0.67	2.13	2.49

	cyclo(Lys(Z)-Pro)$_4$			m.w. 1437.704		
	Pro			Lys		
	C^α	C^β	C^γ	C^α	C^γ	C^ε
	0.14	0.172	0.20*	0.17	0.30	0.42
(e)	0.17		0.38	0.21	0.32	

#) Values with asterisk represent NT_1 of Pro residue taking a cis configuration of peptide bond.

three cyclic octapeptides in CH_3OD or CD_3OD, where N represents the number of protons bound to the carbon. In these solvents all three cyclic octapeptides take C_2-symmetric conformation, and molecular shapes are considered to be similar to each other. The molecular weights of these cyclic octapeptides are ca. 1000, and the measured T_1 values of C^α are in the range of 0.1-0.5 sec. Both indicate that the correlation time τ_c is ca. 10^{-10} sec. This is an extreme motional narrowing, so the relation between T_1 and τ_c can be approximated as following(Eq.(1)) (17). Both the overall rotational motion τ_R and the intramolecular segmental motion τ_g contribute to τ_c(Eq.(2)).Representing the molecule by a sphere of radius a, τ_R can be related to a^3 and the solution viscosity η in an equation, $\tau_R = 4\pi\eta a^3/3kT$.

$$1/NT_1 \simeq \hbar^2\gamma_C^2\gamma_H^2\tau_c/r^6 \qquad (1)$$

$$1/\tau_c = 1/\tau_R + 1/\tau_g \qquad (2)$$

Assuming the proportionality between a^3 and the molecular weight and $\tau_c \simeq \tau_R$, T_1 is inversely proportional to the molecular weight. On the contrary, this inverse proportionality was not found with these cyclic octapeptides; the molecular weight of cyclo(Leu-Pro)$_4$ is 0.86 times as large as that of cyclo(Phe-Pro)$_4$, but in the former NT_1s of C^α are about twise as large as that of the latter. The molecular weight of cyclo-(Lys(Z)-Pro)$_4$ is 1.47 times as large as that of cyclo-(Phe-Pro)$_4$, but NT_1s of C^α are similar. So, the contribution of τ_g to τ_c cannot be neglected. This conclusion is not affected by the concentration of peptides, though NT_1s tend to increase slightly with decreasing concentrations of peptides.

T_1 of Pro-C^{γ} is generally lengthened because of the mobility of pyrrolidine ring. However, in the case of cyclo(Phe-Pro-Gly)$_2$ short T_1s have been reported(18), which are due to the rigidity of pyrrolidine ring associated with the cis configuration of Phe-Pro peptide bonds. In the present case, NT_1s of Pro-C^{β} and C^{γ} connected to the cis peptide bonds of cyclo(Phe-Pro)$_4$ are similar to that of Pro-C^{α}. This indicates that the internal motion of the pyrrolidine ring is so hindered that the overall motion of the residue contributes to the relaxation process. The singularity of available conformation of cyclo(Phe-Pro)$_4$ should be explained in terms of the rigidity of cyclic skeleton caused by severe steric repulsions between side chains. It is therefore suggested that τ_c of cyclo(Phe-Pro)$_4$ is determined mainly by τ_R and the contribution of τ_g is negligible. In the cases of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$, the segmental motion is easier than that in cyclo(Phe-Pro)$_4$, which makes τ_g contribute to τ_c in the former cases. In enniatin B, a motion having the life time less than 10^{-9} sec has been observed by ultrasonic absorption method(19). Therefore, the segmental motions around Leu residue of cyclo(Leu-Pro)$_4$ and Lys(Z) residue of cyclo(Lys(Z)-Pro)$_4$ are more important than the rotational tumblings as compared with the case of cyclo(Phe-Pro)$_4$, and in this sense the formers are more flexible than the latter.

To sum up, the cyclic octapeptides, cyclo(Phe-Pro)$_4$, cyclo(Leu-Pro)$_4$, and cyclo(Lys(Z)-Pro)$_4$ investigated

here, took a C_2-symmetric conformation containing two cis peptide bonds in DMSO-d_6 and CD_3OD. This conformation is proposed to contain two β-turn structures, which are facilitated by the presence of Pro residue. The numbers of available conformation are different among these cyclic octapeptides. The conformational variety is determined by the degree of steric repulsion between side chains. Therefore, it is feasible to control the structure of ring skeleton by choosing properly the steric repulsion between side chains. This steric repulsion was found to influence the segmental mobility, which may be important to determine the ability for complex formation.

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PART II

Interactions of Cyclic Peptides with Small Molecules

Chapter 4

Synthesis, Conformation, and Interactions with Small Molecules of Bis(Cyclic Dipeptides)

INTRODUCTION

A number of studies on the interactions between metal ions and synthetic cyclic peptides as ionophore models have been reported(1). Cyclic tetra-(1), penta-(2), hexa-(1), octa-(3), and decapeptides(3) form complexes with metal ions as a result of intramolecular cooperation of carbonyl groups. In cyclic dipeptides, the carbonyl groups cannot cooperate to form intramolecular complexes. However, cyclo(Sar)$_2$ forms insoluble complexes with various metal ions in ethyl acetate(4). The crystalline complex between cyclo(Sar)$_2$ and $LiClO_4$ has a network structure, in which four carbonyl groups of four cyclic dipeptides coordinate to Li^+(5). Therefore a corresponding bis(cyclic dipeptide) might be an efficient ligand for metal ions because its complexation should be favored over that of simple cyclic dipeptides by the entropy term. We shall call the enhanced complexation caused by intramolecular cooperation of two covalently connected cyclic dipeptide moieties the bis-effect. Such bis-effects have already been postulated and studied in the case of S,S'-bis(cyclo(hemiCys-peptides)) with regard to the complexation of metal ions and their conformations((6-8) and references cited therein).

The bis-effect is expected to depend strongly on the

length and the rigidity of the bridge connecting the two cyclic peptide moieties; so by making a proper choice of the nature of the bridge, one might be able to realize a certain ion selectivity. Peptide complexes of transition-metal ions are particularly interesting, because they might provide models for electron-transporting proteins acting in biological membranes.

In this chapter, the synthesis and complexation with small molecules of bis(cyclic dipeptides), cyclo-(Lys-Pro) cyclo(Glu-Pro) and S,S'-bis(cyclo(hemiCys-Pro)), in which two cyclic dipeptide moieties are linked respectively by an amide bond and a disulfide bond through their side chains, are described. In the complexation with metal ions or dyestuff the bis-effect was manifested, and the mode of complexation was strongly dependent on the character of bridge connecting two cyclic dipeptide moieties.

To elucidate the bis-effect, the conformation of bis(cyclic dipeptide) was analyzed by spectroscopy. Cd and Raman spectra yielded information about the internal rotation around the S,S-bond, and use of a lanthanide probe, $Yb(fod)_3$, enabled the structure of the complex between S,S'-bis(cyclo(hemiCys-Pro)) and metal ions to be determined.

EXPERIMENTAL

Synthesis

The synthetic routes of two bis(cyclic dipeptides) are shown in Figures 1 and 2.

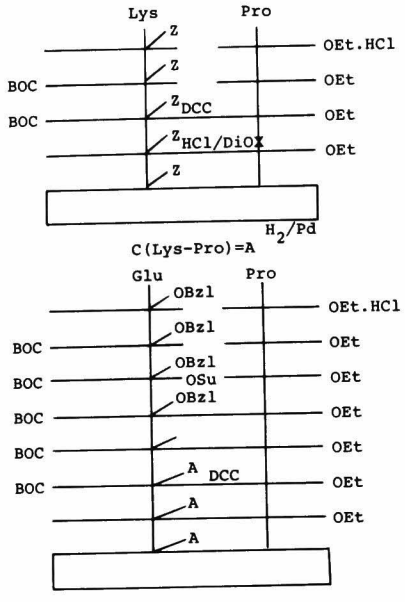

Fig. 1. Synthetic route of cyclo(Lys-Pro) cyclo(Glu-Pro) (bis(I)).

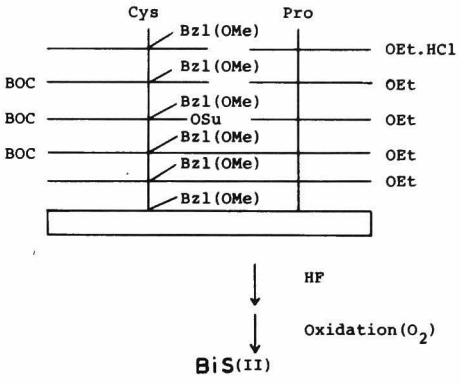

Fig. 2. Synthetic route of S,S'-bis(cyclo(hemiCys-Pro)) (bis(II)).

Cyclo(Lys-Pro) Boc-Glu-Pro-OEt

Boc-Glu-Pro-OEt and cyclo(Lys-Pro) were synthesized by a conventional liquid-phase method. To a solution of Boc-Glu-Pro-OEt (0.25g) and cyclo(Lys(HCl)-Pro) (0.175g) in CH_2Cl_2, triethylamine (93µl) and DCCI (0.138g) were added at 0^oC, and stirred overnight at RT. The solution was evaporated in vacuum and the residue was dissolved in ethyl acetate. Precipitated dicyclohexylurea was filtered off and the filtrate was extracted with water. The product was extracted from the aqueous solution by excess butanol. The butanol phase was dried with Na_2SO_4, evaporated in vacuum, and the residue was recrystallized from ethyl acetate; yield 40%. TLC: R_f(I),

0.18; R_f(II), 0.70. ANAL. Calcd. for $C_{28}H_{45}N_5O_8$: C, 56.28%; H, 7.87%; N, 11.72%. Found: C, 56.63%; H, 7.92%; N, 10.93%.

Cyclo(Lys-Pro) cyclo(Glu-Pro) (Bis(I))

A solution of cyclo(Lys-Pro) Boc-Glu-Pro-OEt (0.3g) in 30 ml of formic acid was stirred for 2 hours at RT. and concentrated under reduced pressure. The oily residue was dissolved in a mixture of 60 ml of 2-butanol and 15 ml of toluene. The solution was refluxed for 2 hours, then concentrated under reduced pressure. The solid residue was recrystallized from ethyl acetate; yield 80%. TLC: R_f(I), 0; R_f(II), 0.63. ANAL. Calcd. for $C_{21}H_{31}N_5O_5 \cdot H_2O$: C, 55.88%, H, 7.32%, N, 15.52%. Found: C, 55.68%; H, 7.11%; N, 16.09%.

Cyclo{Cys(Bzl(OMe))-Pro}

Boc-Cys(Bzl(OMe))-Pro-OEt, synthesized by a conventional liquid-phase method, was treated with 4N HCl/dioxane. The white solid obtained after evaporation was dissolved in methanol containing triethylamine equimloar to the peptide. After standing for 24 hours the solution was concentrated under reduced pressure leaving a white solid, which was dissolved in ethyl acetate, washed with a small amount of water, and dried with Na_2SO_4. After evaporation, the residue was recrystallized from ethyl acetate/2-propanol; yield 61%. TLC: R_f(I), 0.35; R_f(II), 0.75; m.p. 135-136°C. ANAL. Calcd. for $C_{16}H_{20}N_2O_3S$: C, 60.00%; H, 6.25%; N, 8.75%; S, 10.00%. Found: C, 59.91%; H, 6.38%; N, 8.69%; S, 10.13%.

Cyclo(Cys-Pro)

Anisole (1 ml) was added to cyclo{Cys(Bzl(OMe))-

Pro} and HF was led into the apparatus cooled by dry ice/acetone. After stirring for 30 min at 0°C, HF was removed under a reduced pressure at 0°C, and the residue was kept under a reduced pressure for 5 hours. The solid residue was triturated with hexane and dried with NaOH; yield 63%. TLC: R_f(I), 0.45.

S,S'-Bis(cyclo(hemiCys-Pro)) (Bis(II))

CO_2-free air was bubbled for 24 hours through the solution of cyclo(Cys-Pro) in water (pH 6.5). The solution was concentrated to dryness under reduced pressure, and the residual solid was purified by recrystallization. TLC: R_f(I), 0.12. ANAL. Calcd. for $C_{16}H_{22}N_4O_4S_2 \cdot 3/2\ H_2O$: C, 45.16%; H, 6.13%; N, 13.11%; S, 14.99%. Found: C, 45.62%; H, 5.83%; N, 12.75%; S, 15.05%.

RESULTS AND DISCUSSION

Conformation of Bis(cyclic dipeptides)

The conformations available to cyclic dipeptides containing Pro-residue are in general limited because of their bicyclic structure, and the structure of the diketopiperazine backbone is restricted to a bowsprit-boat conformation(9). Figure 3 shows the cd spectra of bis(I) in various solvents. In aqueous solution a positive n-π^* transition and a positive π-π^* transition (long-wavelength lobe) are observed, which indicates that each cyclic dipeptide in bis(I) takes a boat conformation(9). The cd spectra of bis(I) depended on the solvent, the sign of the n-π^* transition being

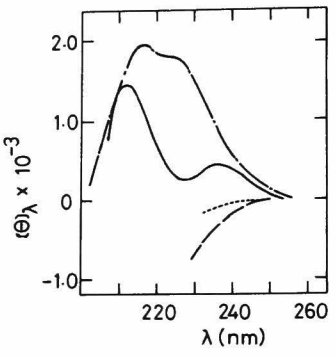

Fig. 3. Cd spectra of cyclo(Lys-Pro)cyclo(Glu-Pro) (bis(I)) in —·—·— H_2O, ——— C_2H_5OH, ····· CH_2Cl_2, and ———$CHCl_3$.

apparently reversed in $CHCl_3$ and CH_2Cl_2. The change of the cd spectra may be caused either by the change of orientation of two cyclic dipeptides or by the change of the state of electronic transition with solvent, allowing for the rigid skeleton of cyclo(Pro-Xyz). The former seems to be the origin of the spectral change in the present case for the following reasons. The temperature dependence of nmr chemical shifts of three amide protons of bis(I) in DMSO-d_6 were measured; these were 3.3 x 10^{-3}, 4.8 x 10^{-3} and 6.0 x 10^{-3} ppm/°C. The rate of exchange with deuterium in methanol-d_4 was slow for the first of three amide protons. These facts suggest that a certain amide proton is involved in the intramolecular hydrogen-bonding. Therefore, the change of cd spectra with solvent should reflect the variation of intramolecular interaction of two cyclic dipeptide moieties of bis(I) as a result of the solvent effect on the hydrogen-bonding. Consequently, in nonpolar solvents intramolecular hydrogen-bonding and amide-amide interaction between two cyclic dipeptide moieties favors a folded structure

of bis(I), while in aqueous solution bis(I) adopts an extended conformation because the intramolecular hydrogen bond is broken.

In the nmr spectra of bis(II) in $CDCl_3$ and DMSO-d_6, only one signal appeared for each proton, so this molecule must adopt a C_2-symmetric conformation on the nmr time scale. The cd spectrum of bis(II) in ethanol showed a positive n-π^* transition and a negative π-π^* transition, which differs from the cd spectra of bis(I). In the case of bis(II) the shortness of the bridge connecting the two cyclic dipeptide moieties results in their proximity allowing sufficient amide-amide interaction. This state is similar to the folded conformation of bis(I) in non-polar solvents. The pattern of cd spectra of bis(II) must have arisen from such a state.

The chemical shift of the amide proton of bis(II) in $CDCl_3$ is 6.47 ppm which is located at a higher magnetic field compared with 7.37 ppm of cyclo(Cys-Pro). This could be accounted for by the magnetic shielding effect of the other diketopiperazine unit, an explanation which is consistent with the previous conclusion about the closeness of the two diketopiperazines of bis(II), although an alternative possibility that the ring skeleton takes a different conformation from the bowsprit-boat conformation is not precluded.

Interaction with Metal Ions

The variation of cd spectrum of bis(I) in ethanol with the addition of $AgClO_4$ is shown in Figure 4. Similar changes of the π-π^* transition are observed with the addition of Na^+ or Ba^{2+} ion, indicating the formation

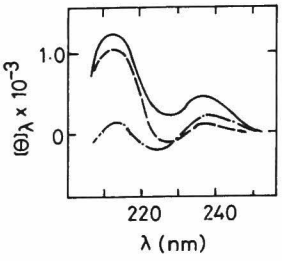

Fig. 4. Cd spectra of cyclo(Lys-Pro) cyclo(Glu-Pro) (bis(I)) in ethanol with or without $AgClO_4$. Molar ratio of $AgClO_4$/bis(I): ——— 0, – – – 1, —·—·— 4.

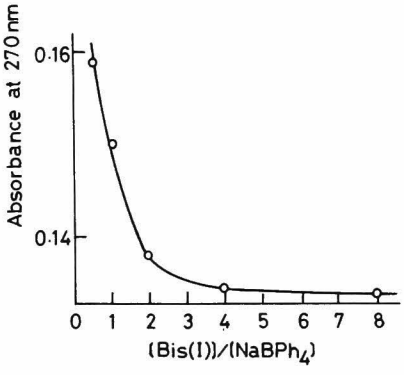

Fig. 5. Extraction of $NaBPh_4$ from water to CH_2Cl_2 with cyclo(Lys-Pro) cyclo(Glu-Pro).

of complex between bis(I) and metal ions.

Figure 5 shows the result of an extraction experiment of sodium tetraphenylborate from water to dichloromethane with bis(I), carried out with varying concentrations of bis(I) in dichloromethane, and the absorption of residual BPh_4^- at 270 nm in aqueous solution was determined. Since the intersection with the abscissa by extrapolation of the initial linear part is two, the stoichiometry of the complex should be peptide/metal ion 2:1. Adopting this stoichiometry, the binding constants were determined for Ba^{2+} and Na^+ complexes from the change of ellipticity in the cd spectra(10). Variation of cd spectra was

Table I

Binding Constants (M^{-2}) of Cyclo(Lys-Pro)cyclo(Glu-Pro) (Bis(I)) and S,S'-bis(cyclo(hemiCys-Pro)) (Bis(II)) with $NaClO_4$ or $Ba(ClO_4)_2$ in Ethanol

	Bis(I)	Bis(II)
Na^+	4.9×10^4	3.2×10^4
Ba^{2+}	2.9×10^5	3.7×10^4

similarly observed with the addition of metal ions to bis(II). Assuming the same stoichiometry of this complex, the binding constants were also obtained with bis(II). The binding constants are given in Table I. For a simple cyclic dipeptide a value of 26-31 M^{-1} was reported for the cyclo(Sar-Gly)/Eu^{3+} complex in $CHCl_3$(1), but the complexation in polar solvents has not been reported. The large binding constants observed with bis(I) in polar solvents may thus be ascribed to the bis-effect.

The complexation of bis(I) is ion-selective; that is, bis(I) is more apt to form a complex with Ba^{2+} than Na^+. On the other hand, bis(II) did not discriminate between Ba^{2+} and Na^+. However, the binding constant cannot be directly related with the nature of peptide ligand without taking into account other factors such as a different charge number between Ba^{2+} and Na^+. This consideration could lead to a different plausible explanation that the structure of bis(II) is so rigid that the cooperative action of the carbonyl groups is more suited for capturing Na^+ than Ba^{2+} and the binding constants appear to be comparable.

The binding constants obseved with bis(I) are in

general larger than those with bis(II). This implies that in the case of bis(II) with a short linkage, conformational adaptation for two cyclic dipeptides to coordinate to metal ion is more difficult than in the case of bis(I) with a long, flexible linkage. For comparison,

$$\text{cyclo(Lys-Try)}\overset{\text{CO(CH}_2\text{)}_4\text{CO}}{\text{ cyclo(Lys-Try)}},$$

which possesses a very long bridge composed of twelve methylene groups and two amide groups, was synthesized and the binding constants for Ba^{2+} and Na^{+} ions were lower than those of bis(I) and bis(II)(11). The very long bridge connecting two cyclic dipeptide moieties made their intramolecular cooperation to coordinate to a metal ion unfavorable. The bis-effect thus depends on the length of bridge, and selective binding of metal ions can be achieved by a proper choice of bridge.

Interactions with Dyestuff and Iodine

With the addition of bis(I) to an aqueous solution of 8-anilino-1-naphthalenesulfonate, the quantum yield of fluorescence of ANS increased and the maximum wavelength of emission shifted to a shorter wavelength (Figure 6). These phenomena were not observed with the addition of cyclo$(Leu)_2$ or bis(II). Therefore, bis(I) stacked an ANS molecule with two cyclic-dipeptide moieties (12). If the same stacking had occurred with two molecules of cyclo$(Leu)_2$, it would have been accompanied by a large entropy loss. In the case of bis(II) the space between the two cyclic dipeptide moieties is too narrow to accommodate a bulky ANS molecule. Dipole-dipole

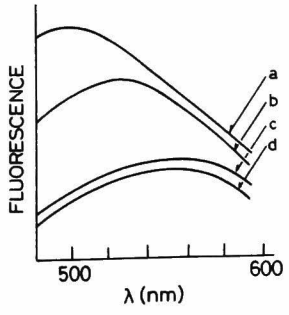

Fig. 6. Fluorescence spectra of 8-anilino-1-naphthalenesulfonate in aqueous solution. a: $2x10^{-5}$M ANS + $4x10^{-4}$M bis(I); b: $2x10^{-5}$M ANS + $1x10^{-4}$M bis(I); c: $2x10^{-5}$M ANS + $4x10^{-4}$M cyclo(Leu)$_2$; d: $2x10^{-5}$M ANS.

interactions and hydrophobic interactions involving the alkyl side chains of Pro- and Lys-residues are supposed to be the attractive force between bis(I) and ANS. The binding constant was estimated to be 1.1 x 10^2 M^{-1} at 20 °C, and increased to 4 x 10^2 M^{-1} on raising the temperature to 50 °C, supporting the explanation that the hydrophobic interaction stabilizes ANS stacked by bis(I).

Complex formation between bis(I) and iodine was illustrated by the UV spectrum. The binding constant was estimated to be 2.1 x 10^2 M^{-1} in $CHCl_3$, which is larger than 8.0 M^{-1} and 1.0 M^{-1} reported for iodine complexes of acetylsarcosine dimethylamide and cyclo(Sar)$_2$, respectively(1). The complex formation between peptides and iodine should be based on the dipole-induced dipole interaction, and the strong complexation of bis(I) with iodine may have resulted from a strong dipolar field produced by the bis-effect.

Structure of Complex between Bis(I) and Metal Ion

Cd

Cd spectra due to S,S-bond of bis(II) in ethanol are shown in Figure 7. The maximum wavelength in this region can be correlated with the dihedral angle θ_s around the S,S-bond. A positive helical sense, that is, $90^{\circ}<\theta_s<180^{\circ}$, was deduced for bis(II)(13). Since no shift of the maximum wavelength was observed with the addition of Ag^+, θ_s does not seem to change upon complexation.

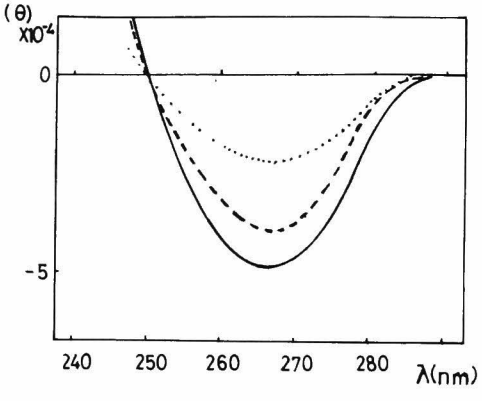

Fig. 7. Cd spectra of S,S'-bis(cyclo(hemiCys-Pro)) (bis(II)) with or without $AgClO_4$ in ethanol. Molar ratio of $AgClO_4$/bis(II): ——— 0, – – – 50, ····· 100.

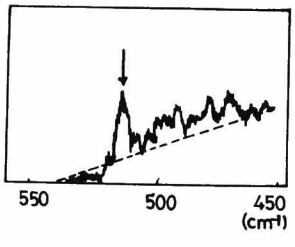

Fig. 8. Raman spectra of S,S'-bis(cyclo(hemiCys-Pro)) (bis(II)) with $Ba(SCN)_2$ in CH_3OH.

Raman Spectrum

In the Raman spectrum of bis(II) in methanol with the addition of Ba^{2+} ion, absorption was observed at 510 cm^{-1}(Figure 8). Miyazawa et al.(14) showed the correlation between the molecular structure around C(β),S-bond of Cys-residue and Raman spectrum to correspond to the complex of bis(II) adopting a gauche form around C(β),S-bond.

Lanthanide Ion Probe Method

Upon adding Yb^{3+}(15), four kinds of molecular species of bis(II), P_f, P_b, $P_f \cdot L_n$, and $P_b \cdot L_n$, are present, but their interconversion is so rapid that the signals are averaged.

$$\begin{array}{ccccc} P_f & + & L_n & \overset{K_f}{\rightleftharpoons} & P_f \cdot L_n \;;\; \Delta\nu_f \\ & \overset{\rightleftharpoons}{} & K_e & & \\ P_b & + & L_n & \overset{K_b}{\rightleftharpoons} & P_b \cdot L_n \;;\; \Delta\nu_b \end{array}$$

P_f=peptide taking a conformation free from bis-effect; P_b=peptide taking a conformation manifesting bis-effect; L_n=lanthanide probe; $\Delta\nu_f$= shift of proton signal in P_f upon binding the probe; $\Delta\nu_b$=shift of proton signal in P_b upon binding the probe

Since bis(cyclic dipeptides) far more easily form complexes with metal ions than simple cyclic dipeptides, $K_e \cdot K_b >> K_f$. Therefore, in the presence of a small amount of Yb^{3+}, $\Delta\nu_b$ should be much larger than $\Delta\nu_f$, and the

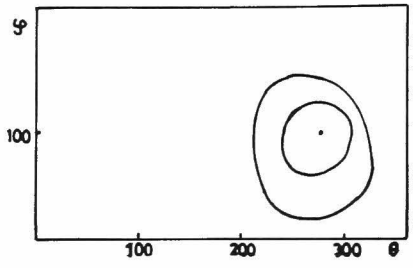

Fig. 9. Map of agreement factor(R). C(α), C(β) and C(γ) of Pro-residue lie on the x,y-plane, and the direction from C(α) to C(β) is positive along the x-axis. θ and φ are the angles of Yb^{3+} from x-axis and z-axis, respectively, on the spherical coordinates with the origin at the oxygen of carbonyl group. The contour lines represent 0.55 and 0.70. The distance between Yb^{3+} and carbonyl oxygen(d) is taken as 3.0 Å.

Table II

Ratio of $Yb(fod)_3$-Induced Shift (QB(I)) of Proton Signals of S,S'-Bis(cyclo(hemiCys-Pro)) (Bis(II))

Proton	QB(I)	Proton	QB(I)
Cys-NH	1.0	Pro-$C^{\delta}H$	0.792
Pro-$C^{\alpha}H$	0.631		0.792
Pro-$C^{\beta}H$	0.408	Cys-$C^{\alpha}H$	0.541
	0.567	Cys-$C^{\beta}H$	0.312
Pro-$C^{\gamma}H$	0.286		0.435
	0.255		

observed relative chemical shifts can be approximated by the ratio of $\Delta\nu_b$, leading to information about the structure of the complex in which two cyclic dipeptide moieties cooperate intramolecularly.

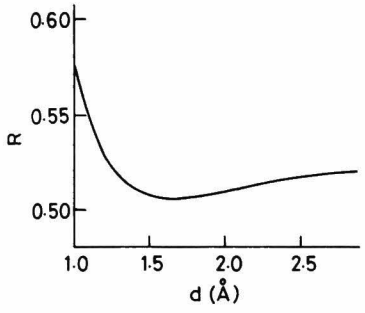

Fig. 10. The dependence of agreement factor (R) on the distance between Yb^{3+} and carbonyl O-atom (d).

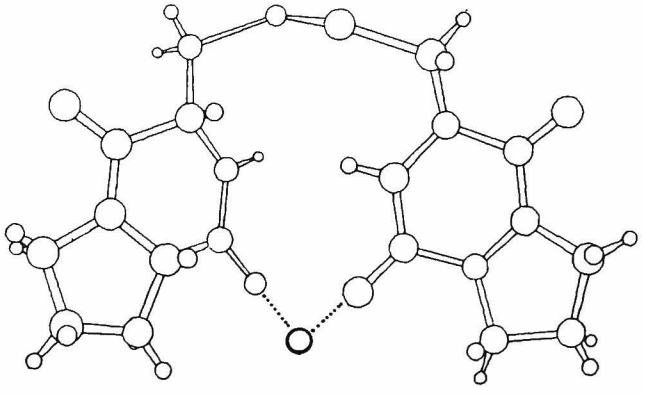

Fig. 11. Proposed conformation of S,S'-bis(cyclo(hemiCys-Pro))/metal ion complex.

The relative chemical shifts of bis(II) caused by the addition of $Yb(fod)_3$ in $CDCl_3$ are summarized in Table II. Assuming the coordination of Yb^{3+} to a carbonyl group of Pro- or Cys-residue and varying the coordination (ϕ,θ,d) of Yb^{3+}, the relative chemical shifts of protons in bis(II) were calculated and compared with the observed values (Table II), and the appropriateness of the calculation was judged by the agreement factor (R) (16). The coordination of each atom of cyclo(Cys-Pro) was taken from the X-ray diffraction analysis of cyclo-(Leu-Pro) (17). Figure 9 shows a contour line map which was calculated assuming the coordination of Yb^{3+} to the carbonyl group of Pro-residue. If Yb^{3+} was assumed to coordinate to the carbonyl group of Cys-residue, R exceeded unity, which is improbable. It was concluded that Yb^{3+} coordinates to the carbonyl group of Pro-residue and the position is determined by $\phi = 100^{\circ}$ and $\theta = 282^{\circ}$.

Figure 10 shows the dependence of R on the distance between the carbonyl oxygen and Yb^{3+}(d). No drastic change of R is seen when d>1.5 Å, thus confirming the above calculation.

The rotation angle χ_1 around C^{α}-C^{β} bond of Cys-residue was determined to be ca. -60°, when the calculated shift ratio of C^{β}-H agreed well with the observed values. The probable conformation of bis(II)/Yb^{3+} complex is shown in Figure 11, drawn with $\chi_1=-50^{\circ}$, $\chi_2=-50^{\circ}$, and the dihedral angle around S,S-bond=140°.

To sum up, cyclic dipeptides are too small to form a

metal ion complex of inclusion type, but with bis(cyclic dipeptides), bis(I) and bis(II), complexes were formed with Ba^{2+} and Na^{+}. A complex of inclusion type surrounded by hydrophobic exterior is believed to work effectively as an ion carrier through a lipid membrane. Bis(cyclic peptides) are very useful for the design of ionophore models.

The bis-effect depends strongly on the nature of the bridge connecting two cyclic moieties and the ability of complex formation decreased in the following order: $-(CH_2)_2-CONH-(CH_2)_4- > -CH_2-S-S-CH_2- > -(CH_2)_4-NHCO-(CH_2)_4-CONH-(CH_2)_4-$. High selectivity in complex formation may be obtained by using and choosing a suitable bis(cyclic peptide).

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Chapter 5

Interactions of Cyclic Hexapeptide Cyclo(Pro-Sar-Sar)$_2$ with Small Molecules in Organic Solvents

INTRODUCTION

Host molecules which can form hydrophobic complexes with hydrophilic guest molecules such as metal ions and amino acid salts are very interesting in view of the ionophore model(1). As described previously, cyclic hexapeptides consisting of N-alkyl α-amino acid may be suitable for this kind of investigation(2). In Chapter 1, cyclo(Pro-Sar-Sar)$_2$ was suggested to possess the conformational multiplicity and flexibility necessary for complexation with metal ions.

In the studies described here, the interactions of cyclo(Pro-Sar-Sar)$_2$ with alkali and alkaline-earth metal ions and ammonium ions were investigated by nmr and cd spectroscopy. In general the size of the hydrophilic cavity formed by a cyclic hexapeptide is not large enough to form a complex of inclusion type. However, cations are expected to be captured by three or four carbonyl groups projecting from the plane of cyclic skeleton in this case.

EXPERIMENTAL

Cyclo(Pro-Sar-Sar)$_2$ was synthesized as described in Chapter 1. α-Amino acid ester hydrochlorides used were identified by elementary analysis, ir, nmr

spectroscopy, and TLC.

Cd spectra were measured at ambient temperatures. Spectroscopic-grade solvents were used without further purification or the solvents were distilled just before use.

RESULTS

Interactions of Cyclo(Pro-Sar-Sar)$_2$ with Alkali and Alkaline-Earth Metal Ions

Cd spectra of ethanolic solutions of cyclo(Pro-Sar-Sar)$_2$ (hereafter abbreviated to c(PSS)$_2$) changed drastically upon addition of $LiClO_4$ (Figure 1), KCl and $Ba(ClO_4)_2$ (Figure 2),and $Cu(ClO_4)_2 \cdot 3H_2O$(Figure 3). C(PSS)$_2$ without the addition of metal salts showed a positive Cotton effect at 212nm. Additions of Li^+, K^+ and Ba^{2+} salts decreased the strength of the Cotton effect and in the case of Ba^{2+} salt addition , the Cotton effect became negative. The addition of excess Ba^{2+} salt caused a white precipitation. However, when Cu^{2+} salt was added, the positive Cotton effect at 212 nm became larger. A negative Cotton effect was observed at 305 nm, and this may be ascribed to the induced circular dichroism at the charge-transfer absorption of Cu^{2+} ion. As reported previously(3), c(PSS)$_2$ in ethanolic solution is a mixture of several conformers in equilibrium. Changes in the cd spectra could be a consequence of variation in conformational equilibrium, and suggests that complex formation of c(PSS)$_2$ with those metal salts takes place.

270 MHz nmr spectra of c(PSS)$_2$ in ethanol solution (N-CH_3 signals only) with and without $LiClO_4$ are shown

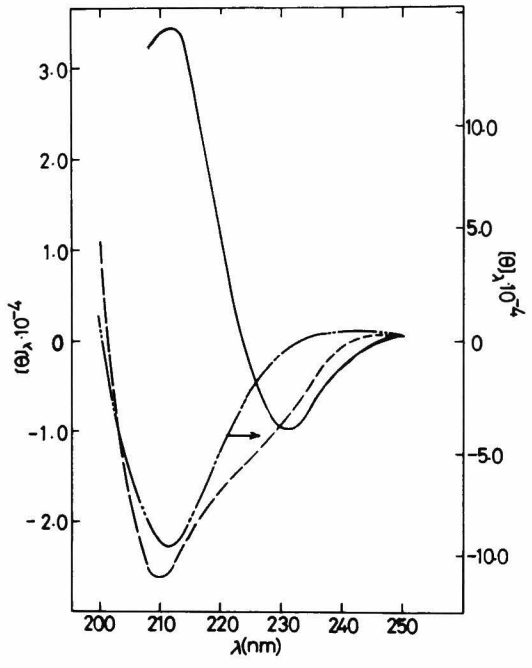

Fig. 1. Change of cd spectrum of cyclo(Pro-Sar-Sar)$_2$ in ethanol solution induced by $LiClO_4$. Molar ratio $(Li^+)/(c(PSS)_2)$: ———, 0; – – –, 3.0; –·–·–, 37.

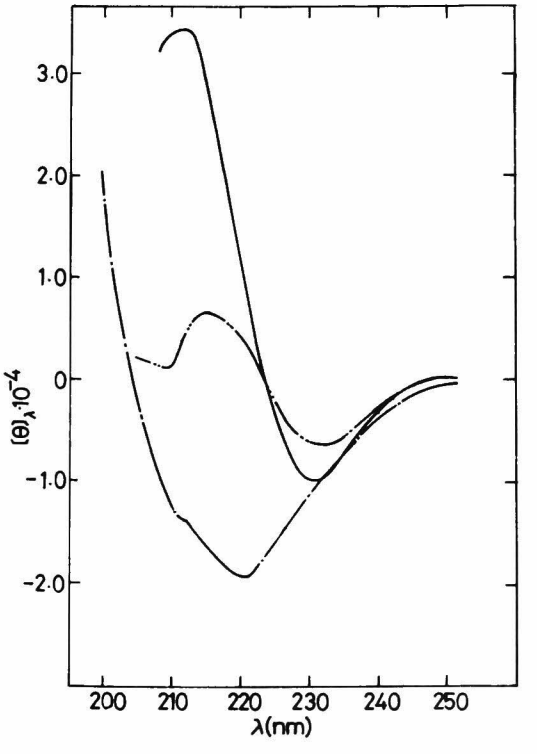

Fig. 2. Change of cd spectrum of cyclo(Pro-Sar-Sar)$_2$ in ethanol solution induced by KCl and $Ba(ClO_4)_2$.
———, without additives;
–·–·–, $(KCl)/(c(PSS)_2)$=2.2;
–·–, $(Ba(ClO_4)_2)/(c(PSS)_2)$ =0.72.

in Figure 4. Many signals were observed for N-CH_3 protons of sarcosyl residues when $LiClO_4$ was absent ((a) in Figure 4). With increasing amounts of added $LiClO_4$ the original signals became weak and four new signals appeared ((b) in Figure 4). Finally, in the presence of excess $LiClO_4$ the signals converged into four of equal intensity ((c) in Figure 4). This fact indicates that $c(PSS)_2$ adopted a unique conformation on complexation with Li^+ ion. This conformation is asymmetric, in which the four peptide bonds involving sarcosyl N-CH_3 groups are trans, trans, trans and cis according to the chemical shifts of the four signals(3).

Changes in the cd spectra were followed as the amount of added Li^+ salt was increased. When the strength of the Cotton effect at 212 nm was plotted against the molar ratio of Li^+ salt and $c(PSS)_2$ a smooth curve without the point of inflexion was obtained. Assuming a 1:1 complex between Li^+ and $c(PSS)_2$ the stability constant of the complex was determined to be 2.3×10^2 M^{-1} according to the molar ellipticity at 212 nm(4).

With regard to the Cu^{2+} salt addition, the same type of plot gave a curve showing a point of inflexion at $(Cu^{2+})/(c(PSS)_2)$ of 0.5. The assumption of 1:1 complex did not lead to a specific stability constant. These findings indicate the simultaneous formation of 1:2 and 1:1 complexes(Figure 5).

Interactions of Cyclo(Pro-Sar-Sar)$_2$ with α-Amino Acid Ester Hydrochlorides

The change in the cd spectrum of $c(PSS)_2$ in a

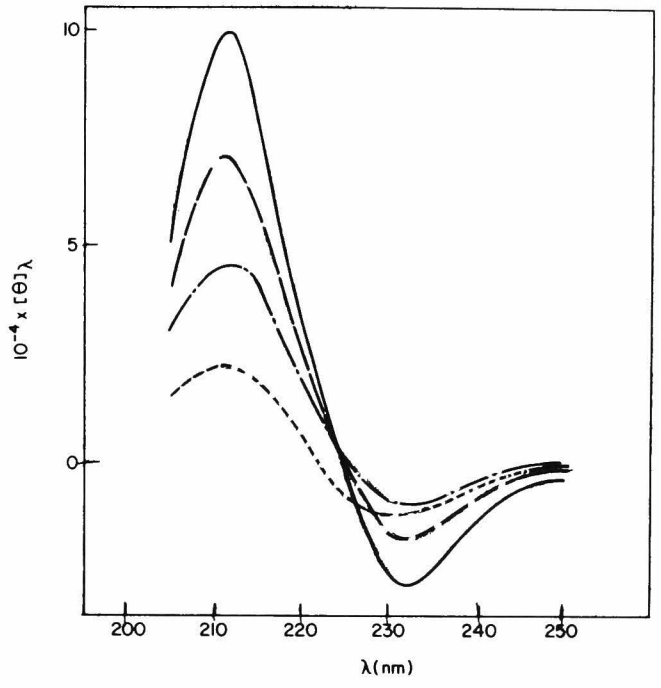

Fig. 3. Change of cd spectrum of $c(PSS)_2$ in ethanol solution induced by $Cu(ClO_4)_2 \cdot 3H_2O$. Molar ratio $(Cu^{2+})/(c(PSS)_2)$: ·····, 0; -·-, 0.37; – – –, 0.93; ——, 1.85.

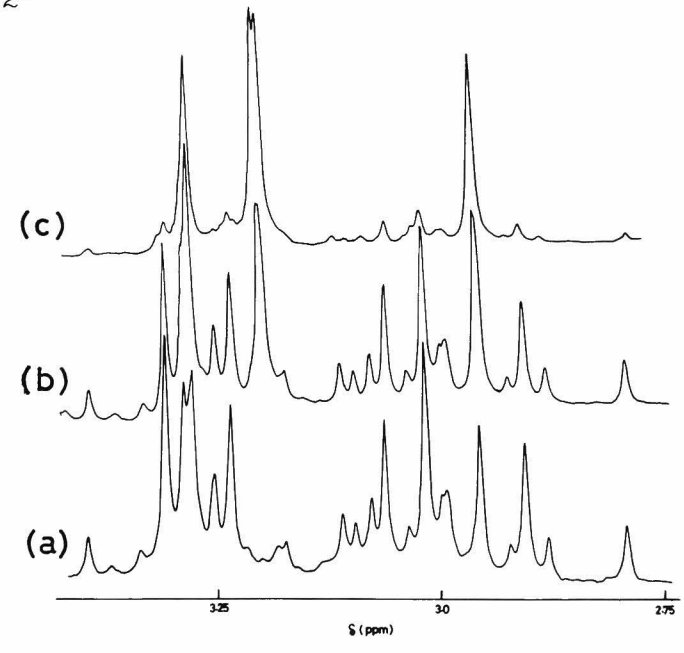

Fig. 4. 270 MHz nmr spectra ($N\text{-}CH_3$ region) of $c(PSS)_2$ in ethanol-d_6 solution with or without $LiClO_4$. Molar ratio $(Li^+)/(c(PSS)_2)$: (a), 0; (b), 0.43; (c),1.9.

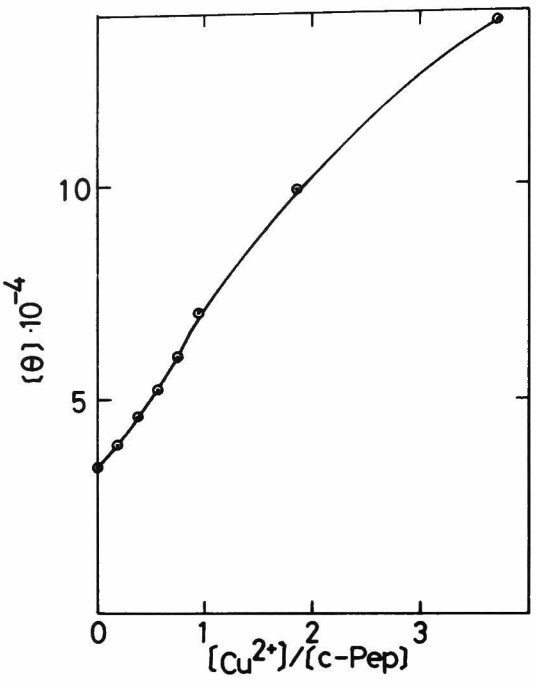

Fig. 5. Change of molar ellipticity at 212 nm of cyclo(Pro-Sar-Sar)$_2$ in ethanol solution induced by $Cu(ClO_4)_2$.

mixture of dioxane/ethanol (4/5 v/v) on addition of racemic α-amino acid ester hydrochloride was investigated. When DL -Val-OEt•HCl was added, there was no marked change in the cd spectrum. However, the addition of DL -Phe-OEt•HCl decreased the strength of the positive Cotton effect at 210 nm. When DL -p-methoxy-phenyl-alanine ethyl ester hydrochloride (DL -p-MeOPhe-OEt•HCl) was added, a similar phenomenon was observed, and a negative Cotton effect reappeared at 227 nm. This new Cotton effect may be due to induced circular dichroism of DL -p-MeOPhe-OEt•HCl. A similar negative Cotton effect due to the induced circular dichroism was also observed when benzonitrile was added. On the basis of these obsevations the above spectral change could be due at least partly to the aromatic-amide interaction(5). (Figures 6, 7, and 8).

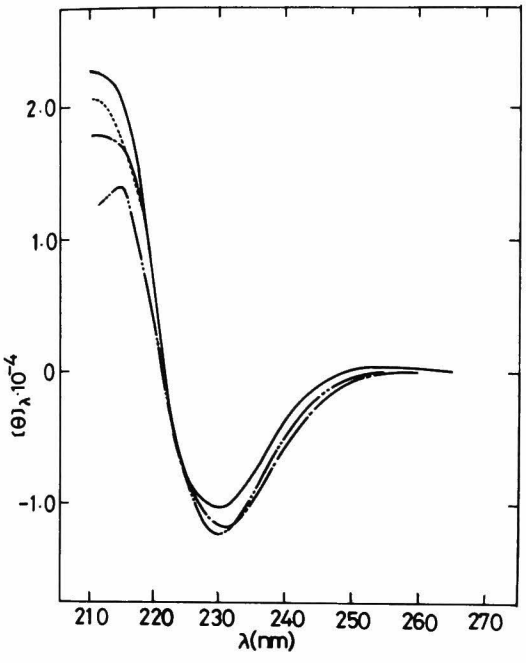

Fig. 6. Change of cd spectrum of $c(PSS)_2$ in dioxane/ethanol (4/5 v/v) mixed solvent induced by α-amino acid ester hydrochloride.——, without additives;·····, (DL -Val-OEt·HCl)/($c(PSS)_2$)=4.9;—·—, (DL -Phe-OEt·HCl)/($c(PSS)_2$)=3.4; —··—, (DL -Phe-OEt·HCl)/($c(PSS)_2$) =5.4.

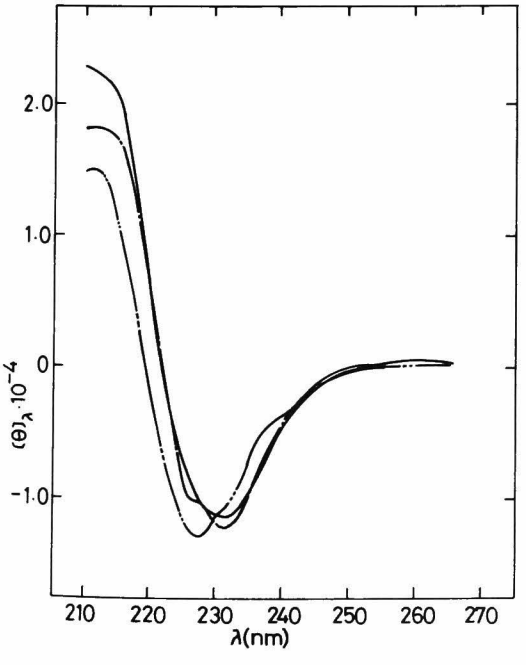

Fig. 7. Change of cd spectrum of $c(PSS)_2$ in dioxane/ethanol (4/5 v/v) mixed solvent induced by DL -p-MeOPhe-OEt·HCl. Molar ratio of (DL -p-MeOPhe-OEt·HCl)/($c(PSS)_2$) :——, 0; —·—, 3.0;—··—, 6.0.

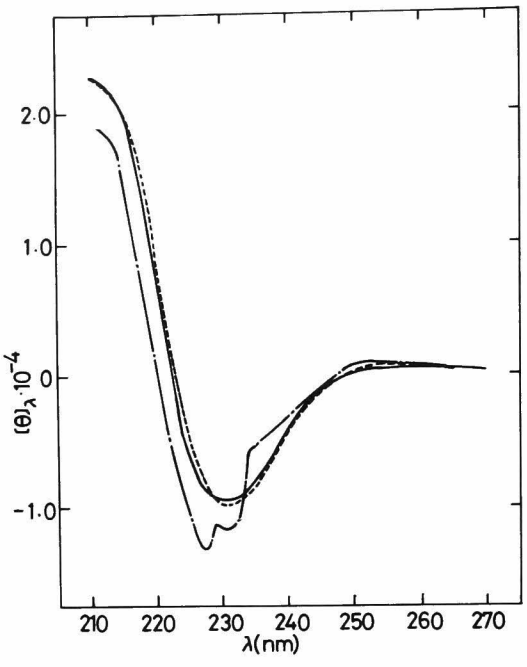

Fig. 8. Change of cd spectrum of $c(PSS)_2$ in dioxane/ethanol (4/5 v/v) mixed solvent induced by aromatic compound.———, without additives;·····, $(C_6H_6)/(c(PSS)_2)=7.9$;—·—, $(C_6H_5CN)/(c(PSS)_2)=5.1$.

100 MHz nmr spectra of $c(PSS)_2$ in $CDCl_3$ solution were investigated with the addition of α-amino acid ester hydrochlorides. The methyl signals of a side-chain isopropyl group of valine ester hydrochloride are shown in Figure 9 in the absence or the presence of $c(PSS)_2$. In Figures 9(b) and (C) two pairs of doublets are seen which have clearly different intensities. Therefore, the α-amino acid ester salts added must be in two different states which are distinguishable on the nmr time scale. One of them should be in a complexed state with $c(PSS)_2$ and the other in the free state. Comparing Figures 9(b) and (c),in both of which racemic α-amino acid ester salt was used, the intensity ratios of two pairs of doublets are nearly equal. But, the chemical-shift difference is large for DL -Val-OBzl·HCl, while it is small for DL -Val-OEt·HCl. Presumably, the benzyl group participates in the binding of DL -Val-OBzl·HCl

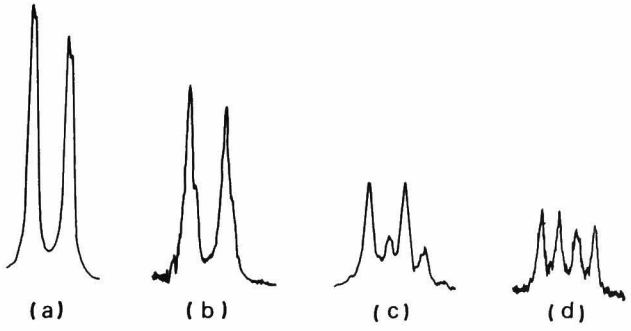

Fig. 9. 100 MHz nmr spectra of side-chain methyl groups of valine ester hydrochloride in $CDCl_3$ solution with or without $c(PSS)_2$. (a), DL -Val-OEt·HCl without $c(PSS)_2$; (b), DL -Val-OEt·HCl with $c(PSS)_2$, molar ratio 1.0; (c), DL -Val-OBzl·HCl with $c(PSS)_2$, molar ratio 0.32; (d), L -Val-OBzl·HCl with $c(PSS)_2$, molar ratio 0.33.

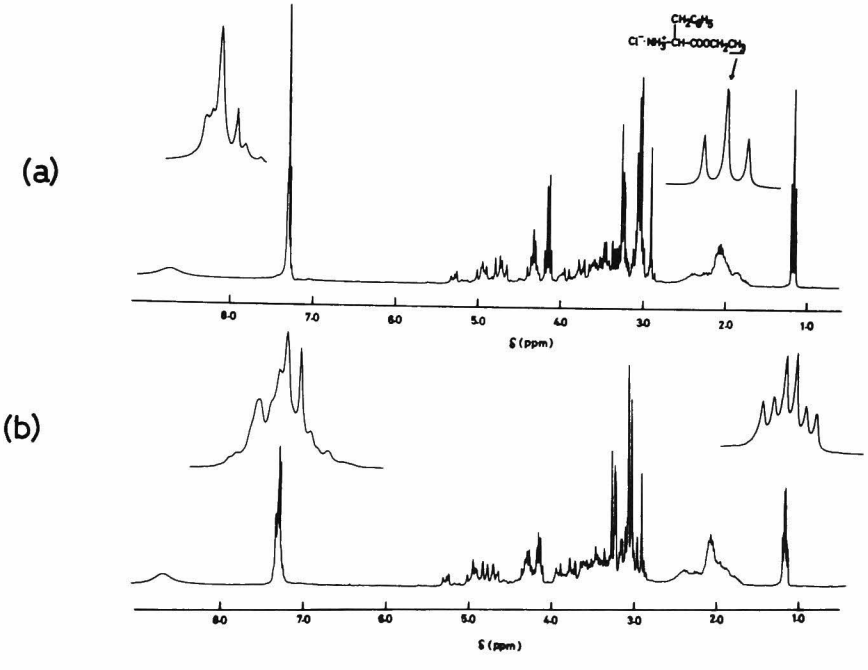

Fig. 10. 270 MHz nmr spectra of $c(PSS)_2$ in $CDCl_3$ solution with Phe-OEt·HCl. (a), (L -Phe-OEt·HCl)/ $(c(PSS)_2)$=1.1; (b), (DL -Phe-OEt·HCl)/$(c(PSS)_2)$=1.0.

by $c(PSS)_2$ and its orientation affects the chemical shift of side-chain methyl groups of bound substrate. Since the fine splitting observed with DL -Val-OEt·HCl in the absence of $c(PSS)_2$ (Figure 9(a)) was not observed in the presence of $c(PSS)_2$, a rapid averaging must occur in both free and bound states. Comparing the complexation with $c(PSS)_2$ of DL -Val-OBzl·HCl (Figure 9(c)) with that of L -Val-OBzl·HCl(Figure 9(d)), two pairs of doublets are again seen, which indicates equilibrium between free and bound substrates. In the present case, however, the intensities of two pairs of doublets are very different with DL -substrate, while they are nearly the same with L -substrate. This indicates that the equilibrium point between free and bound states for L -substrate is different from that for D -substrate. This is a sort of optical resolution.

270 MHz nmr spectra of $c(PSS)_2$ in $CDCl_3$ with Phe-OEt·HCl were investigated. The pattern of CH_3 signals was in a sharp contrast to that of valine ester hydrochloride. When L -Phe-OEt·HCl was added, a single triplet of the methyl signal was observed at 1.18 ppm (Figure 10(a)). This phenomenon indicates a rapid exchange between free and bound L -Phe-OEt·HCl on the nmr time scale. When DL -Phe-OEt·HCl was added, two sets of triplets of the methyl signal were observed which had equal intensities (Figure 10(b)). Since free and bound species are in a state of rapid exchange, the two sets of triplets in Figure 10(b) might be explained in terms of one of two reasons: i) different stabilities of L -Phe-OEt·HCl complex and D -Phe-OEt·HCl complex, ii) different structures of diastereomeric complexes(6).

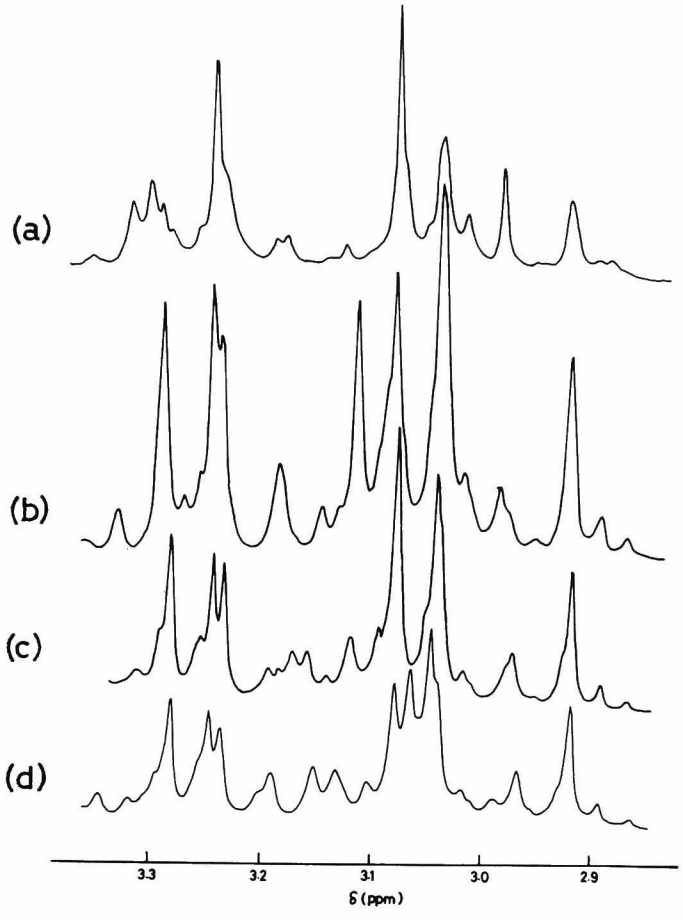

Fig. 11. 270 MHz spectra (N-CH_3 region) of $c(PSS)_2$ in $CDCl_3$ solution with or without DL -Phe-OEt•HCl. Molar ratio (DL -Phe-OEt•HCl)/($c(PSS)_2$): (a), 0; (b), 0.31; (c), 1.0; (d), 1.5.

The solution conformation of $c(PSS)_2$ changed little after the addition of valine ester hydrochloride, but it changed significantly when Phe-OEt•HCl was added, as shown in Figure 11. Only the region of N-CH_3 signal is presented in Figure 11. In addition to a number of N-CH_3 signals before the addition of Phe-OEt•HCl (Figure 11(a)), four new signals appeared after the addition of

DL -Phe-OEt·HCl. One of the four new signals was shifted markedly upfield by the addition of DL -Phe-OEt·HCl. The original N-CH_3 signals were weakened by the addition of DL -Phe-OEt·HCl but still survived in the presence of excess salt. Thus, the addition of α-amino acid ester hydrochloride led to a gradual change in the conformation of $c(PSS)_2$, but it did not converge into a single conformation. This is in marked contrast to the behavior of $c(PSS)_2$ when metal-ion salt is added.

DISCUSSION

The significant change in the cd spectra of $c(PSS)_2$ caused by the addition of alkali and alkaline-earth metal-ion salts and the occurrence of induced circular dichroism of the added salts unambiguously show the formation of stable complex between them. The spectral change induced by Li^+ was different from that of Cu^{2+}. The different behavior reflects the different conformation of $c(PSS)_2$ according to metal ion in the complexed state. This is possible owing to the flexible nature of $c(PSS)_2$ in which the peptide bonds assume either cis or trans configuration(7,8). The conformation of $c(PSS)_2$ complexed with Li^+ ion hardly exists in the uncomplexed state. This also represents the flexibility of $c(PSS)_2$. That conformation of $c(PSS)_2$ in Li^+-complex was asymmetric. This is quite exceptional in view of the fact that most natural cyclic peptides or depsipeptides, such as antamanide and valinomycin (9), and synthetic cyclic peptides such as cyclo(Pro-Gly)$_3$ (10) form symmetrical complexes with metal ions.

The interaction of $c(PSS)_2$ with α-amino acid ester

hydrochlorides was more complicated. On the basis of the observations summarized below, $c(PSS)_2$ is believed to interact with Phe-OEt·HCl through the hydrogen bonding by the ammonium group and the amide-aromatic interaction of the phenyl group.

(1) The interaction of $c(PSS)_2$ with aromatic compound is strong, as is apparent from the induced circular dichroism of benzonitrile.

(2) The change in the cd and nmr spectra upon complexation was more marked with aromatic Phe-OEt·HCl than with aliphatic Val-OEt·HCl.

(3) The significant upfield shift observed with one of the four $N\text{-}CH_3$ signals which appeared after the addition of DL -Phe-OEt·HCl must be due to the aromatic ring-current effect.

(4) Two different states of alkyl protons involved in the ester moiety were observed with DL -Phe-OEt·HCl but not with DL -Val-OEt·HCl. This is probably because the binding is stronger in the former due to the cooperation of two types of interactions.

Another important difference between Val-OEt·HCl complex and Phe-OEt·HCl complex is the exchanging rate of guest molecules between a free state and a bound state. The exchange is rapid in the latter but it is slow in the former on the nmr time scale. If P_o and P_1 represent the conformational states of a host molecule in a free state and a complexed state, respectively, and with A being a guest molecule, the host-guest complexation is expressed by Eqs. (1) and (2), where A* and A discriminate two molecules of the same kind.

$$P_o + A \rightleftharpoons P_1 \cdot A \qquad (1)$$

$$P_1 \cdot A^* + A \rightleftharpoons P_1 \cdot A + A^* \qquad (2)$$

If the conversion of P_o into P_1 accompanies the isomerization of peptide bonds, the activation energy should be high(11). In this case, conformations of $c(PSS)_2$ with isomeric peptide bonds are discriminated by nmr spectroscopy. In the present investigation it was shown that the exchange rate of Eq. (2) depended on the nature of A. This could be explained as follows. $P_1 \cdot A^*$ and A are probably charged and their collision is energetically difficult. However, in the case of Phe-OEt·HCl as a guest molecule, the phenyl group shields the electric charge of ammonium group as a consequence of favorable spatial orientation. The phenyl group may attract other guest molecules by the dipole-dipole interactions. These effects of Phe-OEt·HCl should enhance the exchange of guest molecules between a free state and a bound state. Thus, it is suggested that some spatial arrangement of ammonium group and phenyl group affects not only the exchange of α-amino acid ester hydrochloride (guest molecule) but also the conformational distribution of cyclic peptide (host molecule).

The complex of $c(PSS)_2$ and ammonium salts may not be so stable as metal ion complexes, because the conformation of $c(PSS)_2$ did not converge into a unique one after the addition of excess α-amino acid ester hydrochloride. This is possibly because the spatial fitness of carbonyl groups of C_2-symmetric $c(PSS)_2$ with the C_3-symmetric ammonium group of the guest molecule is not sufficient and the cooperation of carbonyl groups in the binding process is inadequate. In order to

realize a synthetic ionophore which is highly efficient and specific, it is necessary to design a host molecule which carries a number of cooperative groups, possesses a sequence of functional groups to fit with a guest molecule, and undergoes the conformational change with a minimum free-energy change.

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Chapter 6

Interactions of Cyclic Octapeptides, Cyclo(Phe-Pro)$_4$, Cyclo(Leu-Pro)$_4$, and Cyclo(Lys(Z)-Pro)$_4$, with Small Molecules in Organic Solvents

INTRODUCTION

Ionophores, the naturally-occurring biologically active substances, are deeply related to the life by transporting selectively ionic species through the biological membrane or by controlling the membrane potential. A mechanism of carrier-mediated transport by valinomycin has been proposed(1). To realize an ion transport according to the carrier mechanism, the molecule should possess the following basic functions; i) ion binding, ii) selective recognition of ions, iii) high solubility of the complex into membrane, iv) ion releasing.

As pointed in Chapter 5, the high symmetry as well as the molecular flexibility of cyclic peptide is suggested to be essential to the selective ion-binding. Taking this into consideration, a series of cyclic octapeptides cyclo(X-Pro)$_4$, where X represents Phe, Leu or Lys(Z), was synthesized(2). Using these all-L cyclic octapeptides, an efficient complex formation with metal ions is expected due to an effective cooperation of four carbonyl groups when the cyclic octapeptides take a C_4-symmetric conformation. Investigations described in Chapter 3 suggested that the conformational properties

influence the complex formation of the cyclic octapeptides with small molecules.

In this chapter, the complex formation of these cyclic octapeptides with alkali and alkaline earth metal ions was investigated. Special attentions were focused on the equilibrium and the kinetic aspects of the complex formation and the solvent effect, and the relation between the conformational flexibility and the ability of complex formation of cyclic peptides was discussed.

EXPERIMENTAL

The syntheses of the cyclic octapeptides have been described previously(2). The metal-ion salts used were reagent-grade and used without further purification. Spectral-grade solvents or freshly distilled solvents were used.

Complexes were formed by adding the salt solution to the peptide solution. Unless otherwise stated, the whole system was homogeneous.

RESULTS AND DISCUSSION

Complex Formation in Alcoholic Solution

No change of circular dichroism spectrum of cyclo-$(Phe\text{-}Pro)_4$ in ethanol/water mixture was observed with the addition of perchlorates of Li^+, Na^+, Cu^{2+} and Ba^{2+}, and chlorides of K^+ and Cs^+. No change in the ^{13}C nmr of cyclo$(Phe\text{-}Pro)_4$ was also detected with the addition

of $Ba(ClO_4)_2$ in CD_3OD/D_2O (95/5 v/v) mixture. These facts indicate that cyclo(Phe-Pro)$_4$ does not form complexes with metal ions in the above solvents. The previous study showed that cyclo(Phe-Pro)$_4$ was a rigid molecule to take a C_2-symmetric conformation containing two cis peptide bonds in various solvents(2). This rigidity is a likely reason for cyclo(Phe-Pro)$_4$ not to adjust the conformation to one suitable for binding metal ions.

In case of cyclo(Leu-Pro)$_4$, no sign of complex formation with perchlorates of Li^+, Mg^{2+} and Cu^{2+} was observed by cd spectra in CH_3OH/H_2O (95/5 v/v) mixture. However, by the additions of $Ca(ClO_4)_2$, $Ba(ClO_4)_2$, and KCl, cd spectra changed due to the complex formation as shown in Figure 1. Figure 2 shows the variation of ^{13}C nmr spectra of cyclo(Leu-Pro)$_4$ with the addition of $Ba(ClO_4)_2$. It shows a gradual change of the conformation of cyclo(Leu-Pro)$_4$ from a C_2-symmetric one in a free state into a C_4-symmetric one in a complexed state. All peptide bonds involving the N-terminal of Pro residues in the complex were determined to be cis on the basis of the chemical shifts of C^β (32.8 ppm) and C^γ (21.7 or 21.4 ppm) of Pro residue(3,4).

No change of cd spectra of cyclo(Lys(Z)-Pro)$_4$ in ethanol/water (95/5 v/v) mixture was observed with the addition of perchlorates of Li^+, Na^+, Mg^{2+} and Cu^{2+} and CsCl. However, by the addition of the perchlorates of Ag^+, Ca^{2+} and Ba^{2+} and KCl, cd spectrum changed as shown in Figure 3. The variation of ^{13}C nmr spectrum of cyclo(Lys(Z)-Pro)$_4$ with the addition of $Ba(ClO_4)_2$ indicated that the C_2-symmetric conformation in a free state changed into a C_4-symmetric one containing four cis peptide bonds in a complexed state. The pattern of spectral change is very similar to that observed with

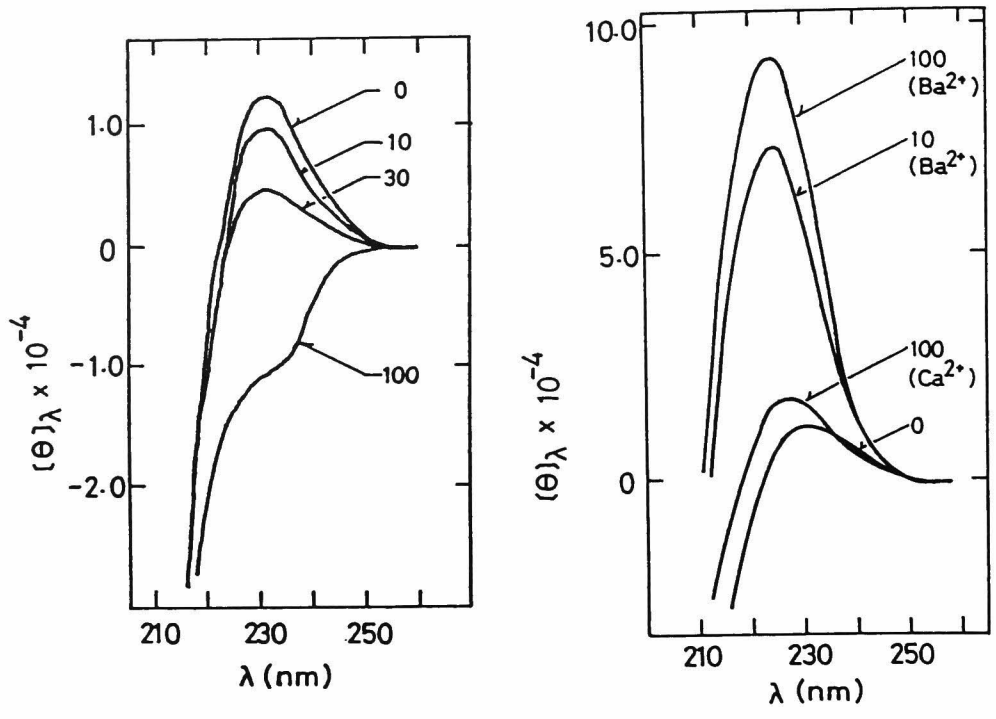

Fig. 1. Cd spectra of cyclo(Leu-Pro)$_4$. (a) in 86% CH_3OH with or without KCl, (cyclo(Leu-Pro)$_4$)=9.3 x 10^{-4} M^{-1}; (b) in 95% CH_3OH with or without $Ba(ClO_4)_2$ or $Ca(ClO_4)_2$, (cyclo(Leu-Pro)$_4$)=8.8 x $10^{-4}M^{-1}$. Numbers represent the molar ratio of metal salt added against cyclo(Leu-Pro)$_4$.

cyclo(Leu-Pro)$_4$.

It was shown in the present investigation that cyclo-(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ formed complexes selectively with Ba^{2+}, Ca^{2+} and K^+. Assuming a 1:1 complex formed, the binding constants were determined from the change of molar ellipticity in cd spectra(8) and are shown in Table I. As shown by ^{13}C nmr spectra, cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ changed their conformations by the cis/trans isomerization of peptide bonds of N-substituted amino acid residues into highly symmetric conformations in which four carbonyl groups preferably cooperate to capture a metal ion.

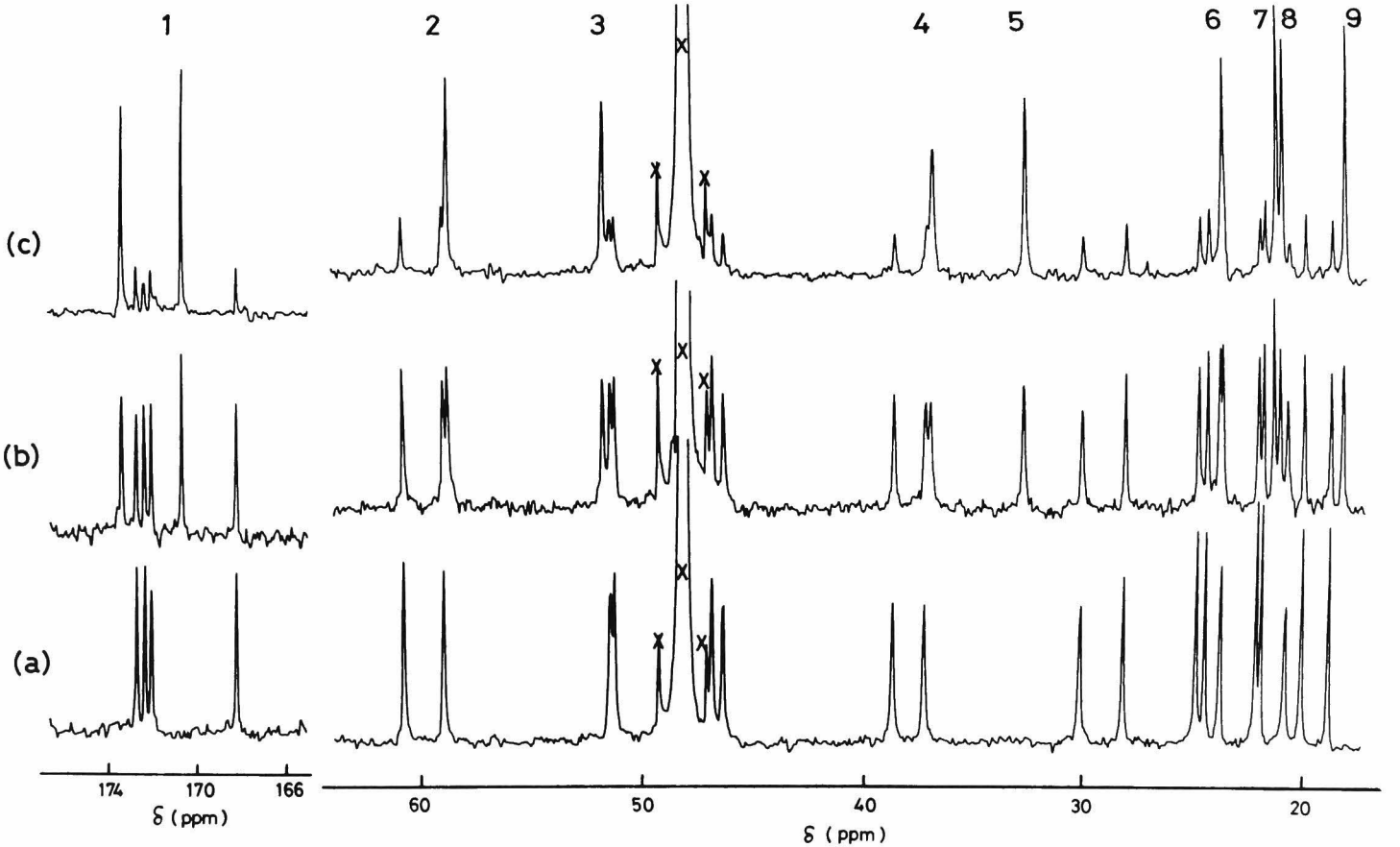

Fig. 2. ^{13}C nmr spectra of cyclo(Leu-Pro)$_4$ in 95% CH_3OD with or without $Ba(ClO_4)_2$, [cyclo(Leu-Pro)$_4$] = 6.2 x 10^{-2} M^{-1}. The molar ratio of $Ba(ClO_4)_2$ against cyclo(Leu-Pro)$_4$ is (a), 0; (b), 0.44; (c), 0.81. 1, C=O; 2, Pro-C^{α}; 3, Leu-C^{α}; 4, Leu-C^{β}; 5, Pro-C^{β}; 6, Leu-C^{γ}; 7,8, Leu-C^{δ}, Pro-C^{γ}; 9, Leu-C^{δ}.

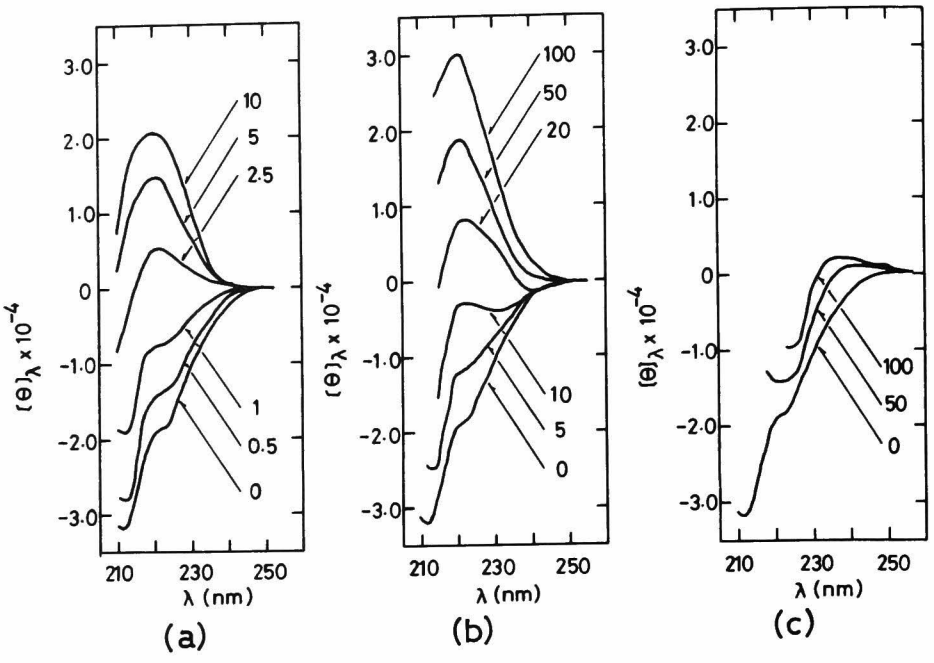

Fig. 3. Cd spectra of cyclo(Lys(Z)-Pro)$_4$ in 95% ethanol with or without (a) $Ba(ClO_4)_2$, (b) $Ca(ClO_4)_2$, (c) $AgClO_4$. (cyclo(Lys(Z)-Pro)$_4$) = $4.0 \times 10^{-4} M^{-1}$. Numbers represent the molar ratio of metal salt added against cyclo(Lys(Z)-Pro)$_4$.

TABLE I

Stability Constants of Cyclic Octapeptide-Metal Salt Complex (M^{-1})

	$Ba(ClO_4)_2$	$Ca(ClO_4)_2$	KCl
Cyclo(Leu-Pro)$_4$	4.2×10^2 [a]	very low	~ 3 [b]
Cyclo(Lys(Z)-Pro)$_4$	1.3×10^3 [c]	1.3×10^2 [c]	~ 3 [b]

[a] 95% CH_3OH [b] 86% CH_3OH [c] 95% C_2H_5OH

These cyclic peptides did not form complexes efficiently with monovalent metal ions and ions having a small radius. Steric repulsion between bulky side chains restricts possible conformational changes to bind small ions, and hence accounts for the ion selectivity. The binding constants of cyclo(Lys(Z)-Pro)$_4$ are larger than those of cyclo(Leu-Pro)$_4$. The conformational change of the former for complexation seems to be easier than that of the latter because of its smaller steric repulsion between side chains.

Complex Formation in Acetonitrile

It was shown in Chapter 3 that the conformational distribution of cyclic octapeptides in free states depended strongly on the nature of solvent. This fact implies that the complex formation of cyclic peptides will be affected by solvent.

Cd spectra of cyclo(Phe-Pro)$_4$ in acetonitrile in the presence of $Ba(ClO_4)_2$ are shown in Figure 4(a). Cd spectra changed with the addition of Ba^{2+} in this solvent. ^{13}C nmr spectral change of cyclo(Phe-Pro)$_4$ is shown in Figure 5. Cyclo(Phe-Pro)$_4$ in acetonitrile took a C_2-symmetric conformation containing two cis peptide bonds in a free state. With the addition of a half equivalent amount of Ba^{2+} three kinds of conformations appeared, which were a C_2-symmetric one, a C_4-symmetric one with all-trans peptide bonds, and a C_4-symmetric one with all-cis arrangements of Phe-Pro peptide bonds. Adding further amount of Ba^{2+} ion, a C_4-symmetric conformation with all-trans peptide bonds became predominant. Therefore, cyclo(Phe-Pro)$_4$ was shown to form a complex with Ba^{2+} in acetonitrile accompa-

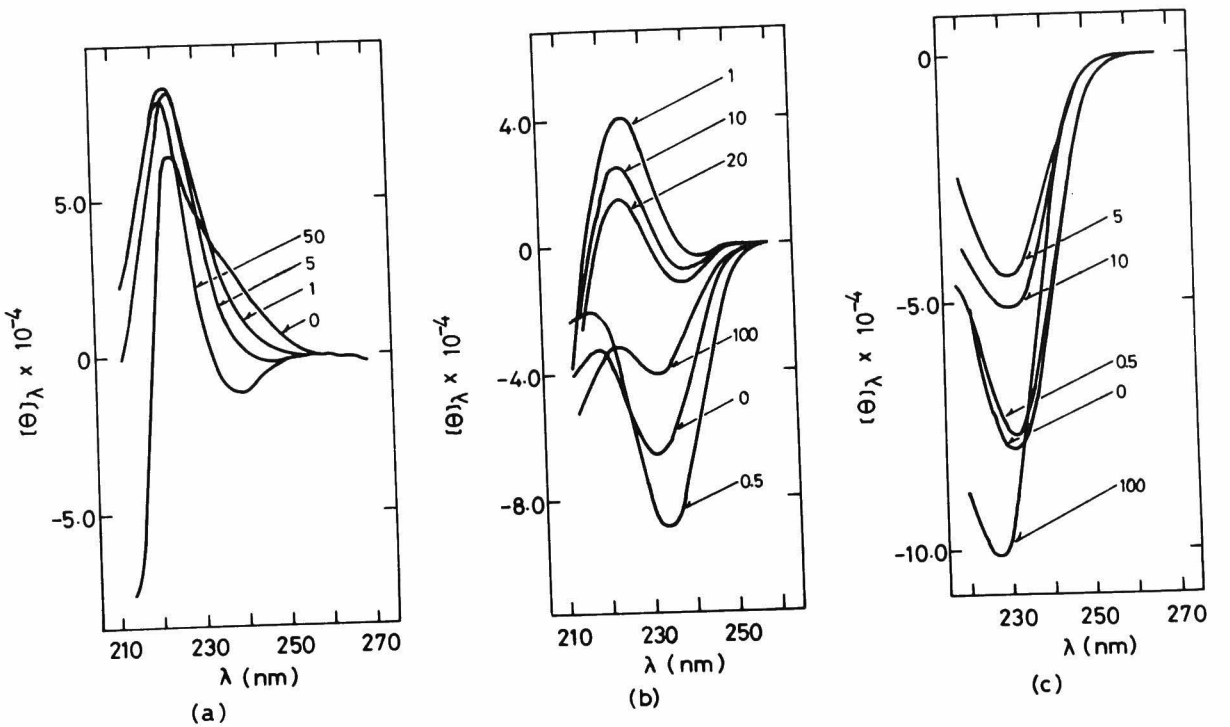

Fig. 4. Cd spectra of cyclic octapeptides in CH_3CN with or without $Ba(ClO_4)_2$. (a) cyclo(Phe-Pro)$_4$, 3.7 x 10^{-4} M^{-1}; (b) cyclo(Leu-Pro)$_4$, 7.6 x 10^{-4} M^{-1}; (c) cyclo(Lys(Z)-Pro)$_4$, 6.0 x 10^{-4} M^{-1}; numbers represent the molar ratio of metal salt added against cyclic octapeptide.

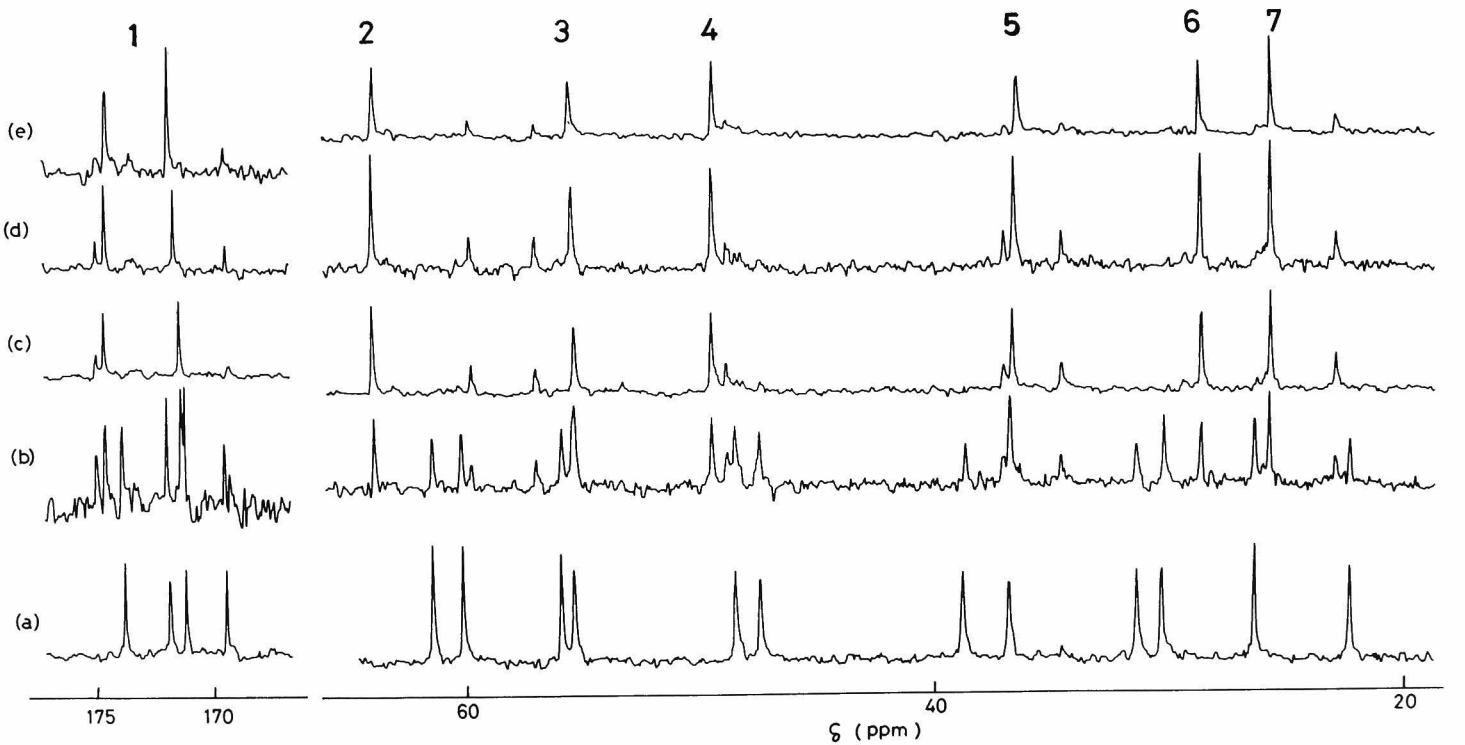

Fig. 5. ^{13}C nmr spectra of cyclo(Phe-Pro)$_4$ in CD_3CN with or without $Ba(ClO_4)_2$. [cyclo(Phe-Pro)$_4$] = 3.6 x 10^{-2} M^{-1}. The molar ratio of $Ba(ClO_4)_2$ against cyclo(Phe-Pro)$_4$ is (a), 0; (b), 0.5; (c). 1.0; (d), 1.5; (e), 2.5. 1, C=O; 2,Pro-C^{α}; 3, Phe-C^{α}; 4, Pro-C^{δ}; 5, Phe-C^{β}; 6, Pro-C^{β}; 7, Pro-C^{γ}.

nied by the isomerization of peptide bonds.

The addition of $Ba(ClO_4)_2$ to cyclo(Leu-Pro)$_4$ in acetonitrile decreased the strength of the negative Cotton effect around 230 nm region at first, and then increased the strength to a positive value (Figure 4(b)). Further addition of the salt decreased the strength to a negative value. The whole change can be interpreted in terms of the formation of three kinds of species. The change in ^{13}C nmr spectrum is shown in Figure 6. The addition of a half equivalent amount of $Ba(ClO_4)_2$ changed the conformation of cyclo(Leu-Pro)$_4$ from a mixture of a C_2-symmetric one and a C_4-symmetric one with all-trans peptide bonds into the latter. Adding more metal salt, a C_4-symmetric conformation with four cis Leu-Pro peptide bonds newly appeared. The correspondence between the cd and the nmr behaviors indicates that the negative Cotton effects around 230 nm region is assignable to a C_4-symmetric conformation with all-trans peptide bonds and the positive Cotton effect to a C_4-symmetric conformation with all-cis arrangements of Leu-Pro peptide bonds. Although some reserve should be made on the above conclusion because of the concentration difference between the cd and the nmr measurements, the relationship established between the Cotton effect around 230 nm region and the type of conformation should be firm and applicable to other solvent systems and to other cyclic octapeptides such as cyclo(Lys(Z)-Pro)$_4$. For example, a C_4-symmetric conformations of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$, which have all-cis arrangements of X-Pro peptide bonds, exhibited a positive Cotton effect around 230 nm region in alcoholic solution (see Figures 1 and 3). Since the present cyclic octapeptides differ from each other only with the side chains of X-residue, the arrangement

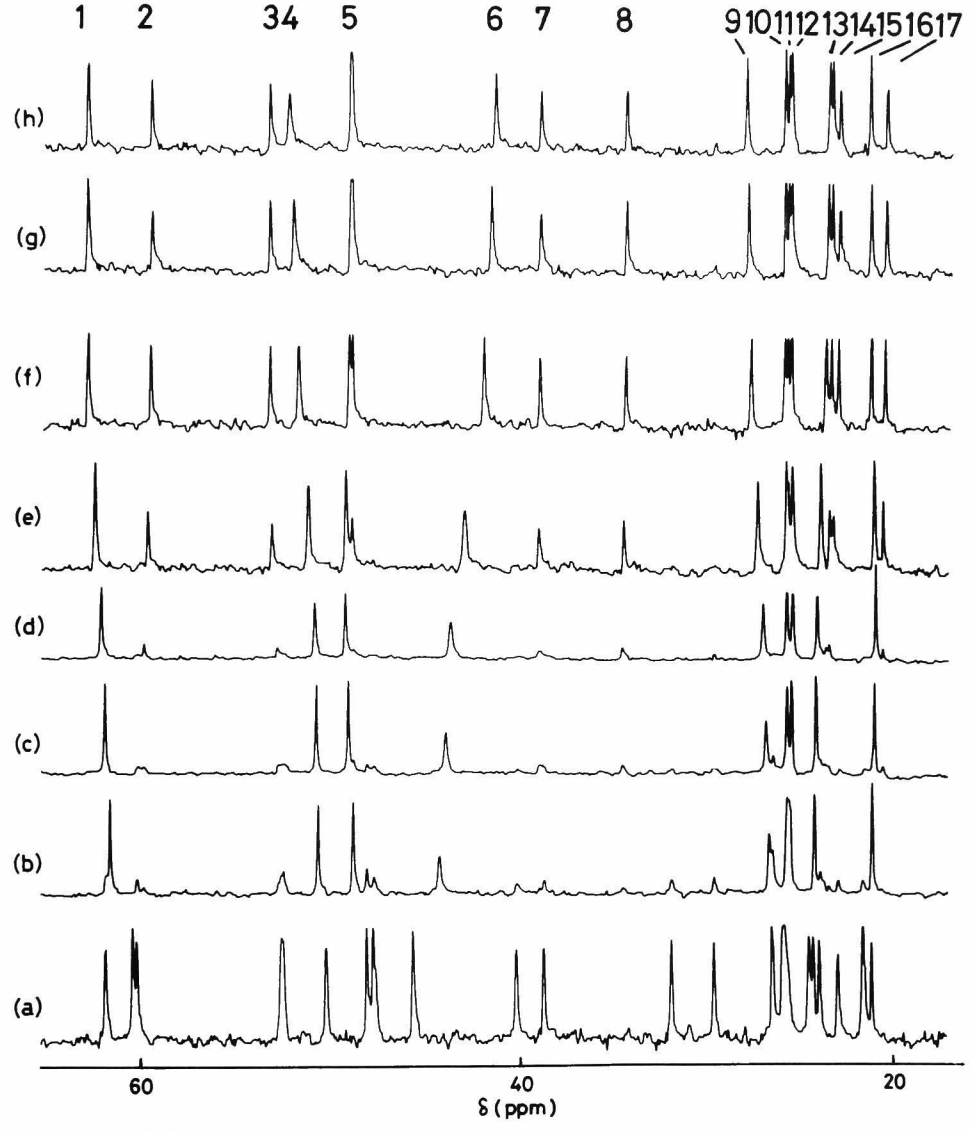

Fig. 6. ^{13}C nmr spectra of cyclo(Leu-Pro)$_4$ in CD_3CN with or without $Ba(ClO_4)_2$, [cyclo(Leu-Pro)$_4$]=4.2x10^{-2} M^{-1}. The molar ratio of $Ba(ClO_4)_2$ against cyclo(Leu-Pro)$_4$ is (a),0; (b),0.2; (c),0.3; (d),0.4; (e),0.6; (f),0.8; (g),1.0; (h),1.4. 1,Pro-C^{α}(t);2,Pro-C^{α}(c);3, Leu-C^{α}(c);4,Leu-C^{α}(t);5,Pro-C^{δ}(t,c);6,Leu-C^{β}(t);7,Leu-C^{β}(c);8,Pro-C^{β}(c);9,Pro-C^{β}(t);10,11,12,Leu-C^{γ}(t,c),Pro-C^{γ}(t);13,Leu-C^{δ}(t);14,15,Leu-C^{δ}(c),Pro-C^{γ}(c);16,Leu-C^{δ}(t);17,Leu-C^{δ}(c). t and c represent all-trans and four cis C_4-symmetric conformations, respectively.

of peptide bond of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$, which determines the pattern of cd spectra, should be the same for the same conformational symmetry.

Next, the stoichiometry of the Ba^{2+} complex of cyclo(Leu-Pro)$_4$ was investigated. Figure 7 shows the change of chemical shifts of carbonyl carbons of cyclo-(Leu-Pro)$_4$ by the addition of increasing amount of $Ba(ClO_4)_2$. The chemical shifts of carbonyl carbons arisen from the C_4-symmetric conformation with all-trans peptide bonds changed greatly with the addition of metal salt. These changes of chemical shifts were considered to be due to metal-ion binding. The interconversion between a free and a complexed state is so rapid on the nmr time scale that only averaged signals were observed. With the addition of less than a half equivalent amount of $Ba(ClO_4)_2$, the signals located at lower magnetic field shifted mainly, which indicates the complexation on one side of cyclic skeleton. With more than a half equivalent amount of salt, the signals located at higher magnetic field shifted extensively, which indicates the complexation on both sides of cyclic skeleton. So, the C_4-symmetric conformer with all-trans peptide bonds seems to form complexes having peptide/Ba^{2+} molar ratio of 2 (peptide sandwich) and 0.5 (metal-ion sandwich). A C_4-symmetric conformer with all-cis arrangements of Leu-Pro peptide bonds which also appeared by the addition of $Ba(ClO_4)_2$ seems to form a 1:1 complex in analogy with the complexation in alcoholic solution.

Addition of Ba^{2+} to cyclo(Lys(Z)-Pro)$_4$ changed the cd spectrum as shown in Figure 4(c). Two types of species are present. The conformation which appeared with the addition of a small amount of metal salt is considered to be a C_4-symmetric one with all-cis arrangements of

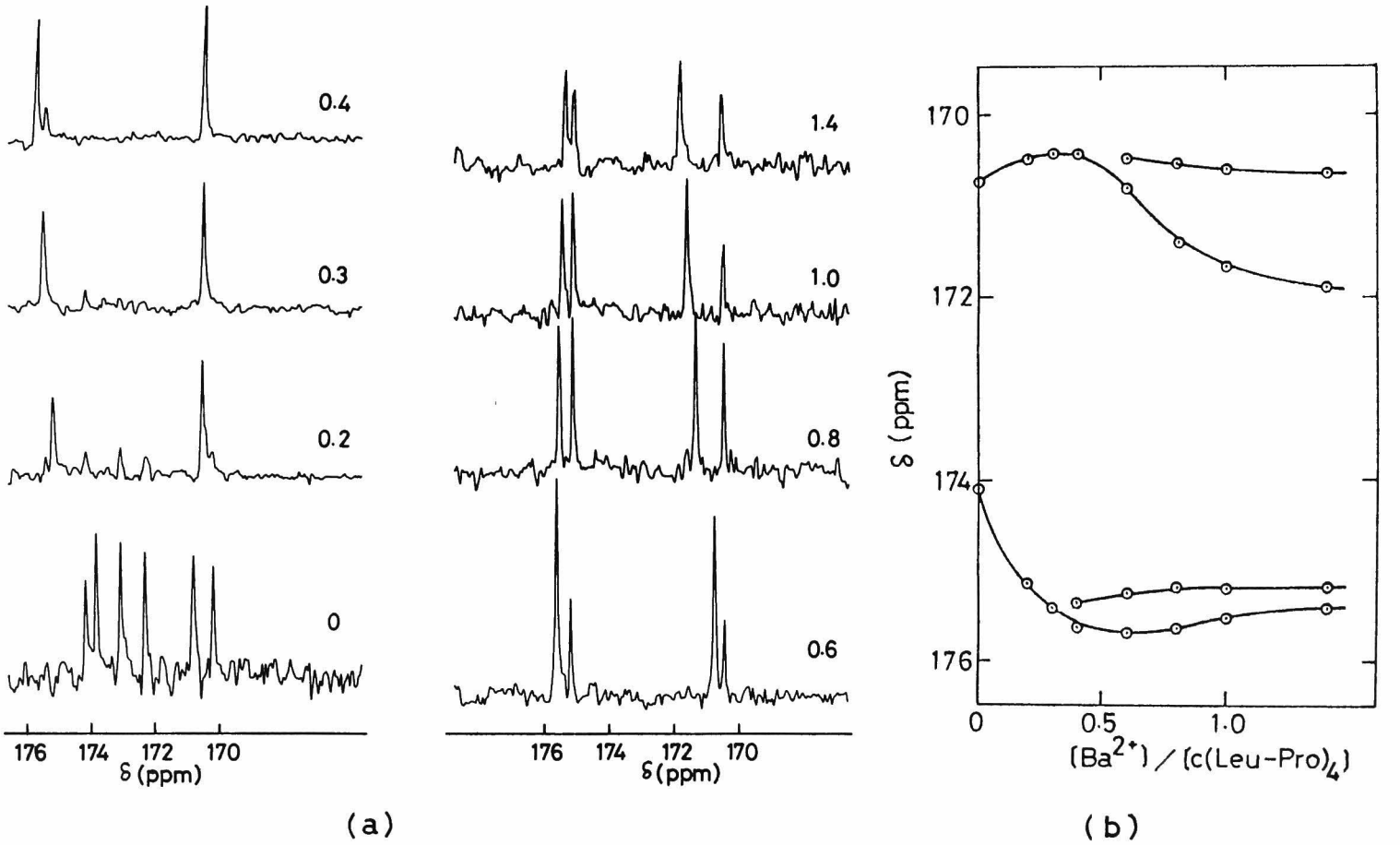

Fig. 7. (a) ^{13}C nmr spectra and (b) chemical shifts of C=O signals of cyclo(Leu-Pro)$_4$ with or without $Ba(ClO_4)_2$ in CD_3CN. Numbers represent the molar raio of metal salt added against cyclo(Leu-Pro)$_4$.

Lys(Z)-Pro peptide bonds from ^{13}C nmr spectra. However, the details of this complexation are unclear because a further addition of the metal salt yielded a precipitation.

Figure 8 shows the changes of cd spectra with the addition of KCl to three kinds of cyclic octapeptides in 99% acetonitrile/H_2O mixture. The addition of the metal salt caused a little change of the spectra of cyclo(Phe-Pro)$_4$. On the other hand, it decreased the strength of the negative Cotton effect of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$. Cd spectra at high salt concentrations were not recorded on account of the low solubility of KCl, and the equilibrium constant of the complex formation was not determined. However, judging from the large spectral change, the binding constants between cyclo(Leu-Pro)$_4$ or cyclo(Lys(Z)-Pro)$_4$ and K^+ seem to be quite large.

The features of complex formations of cyclic octapeptides in acetonitrile are summarized as follows; i) appearance of new all-trans C_4-symmetric conformation which is absent in alcohol, ii) formation of 2:1 and 1:2 peptide/Ba^{2+} complexes, iii) higher binding constants than those in alcohol, iv) conformational change of rigid cyclo(Phe-Pro)$_4$ with the addition of Ba^{2+}. Therefore, it can be concluded that not only the stereochemical fit between a cyclic peptide and a guest molecule but also the solvation of the complex formed are important in the complexation.

Kinetics of Complexation between Cyclic Octapeptides and Metal Salts

For the investigation of the ion transport through the membrane, the formation and the dissociation constants

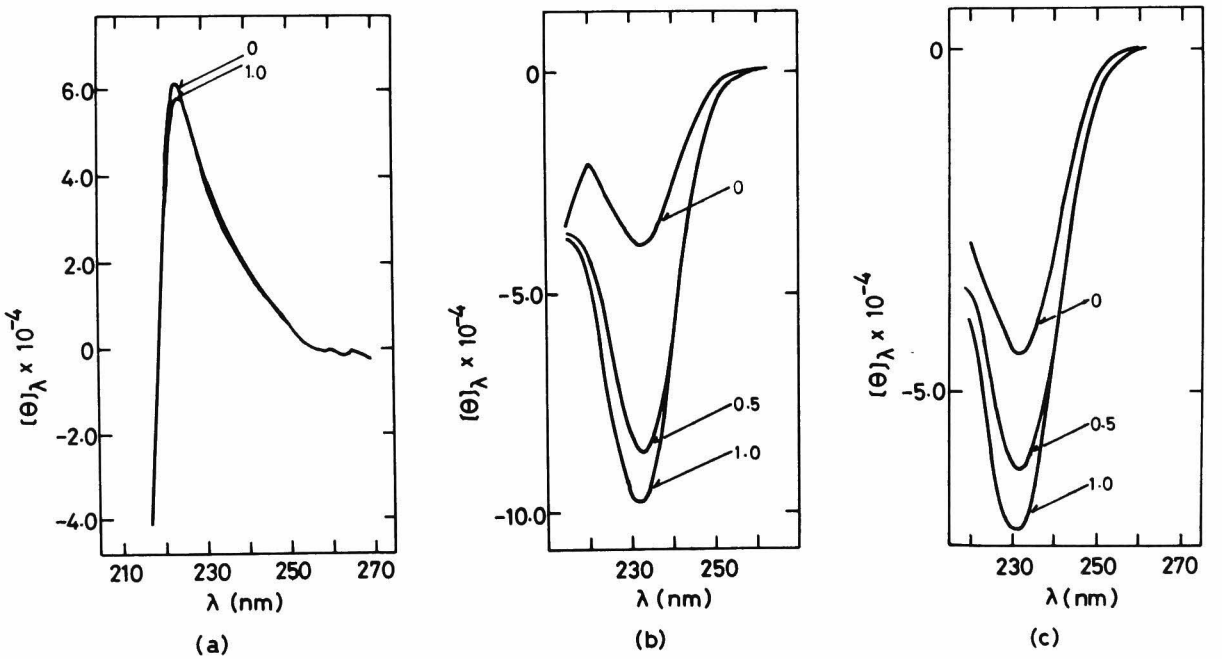

Fig. 8. Cd spectra of cyclic octapeptides in 99% CH_3CN with or without KCl. (a) cyclo(Phe-Pro)$_4$, 5.2 x $10^{-4}M^{-1}$; (b) cyclo(Leu-Pro)$_4$, 1.1 x 10^{-3} M^{-1}; (c) cyclo(Lys(Z)-Pro)$_4$, 8.3 x 10^{-4} M^{-1}. Numbers represent the molar ratio of metal salt added against cyclic octapeptides.

as well as the binding constant are necessary to be determined.

Figure 9(a) shows the time-resolved ellipticity at 225 nm of cd spectrum of cyclo(Leu-Pro)$_4$ in 95% CH_3OH/H_2O mixture with the addition of Ba^{2+}. Under these conditions only a 1:1 complex was formed. Assuming the equilibrium of complex formation as in Eq. (1), Eq. (2) is deduced;

$$P_f + M \underset{k_b}{\overset{k_f}{\rightleftharpoons}} P_c \cdot M \qquad (1)$$

$$\frac{1}{(A - B)} \ln \frac{(\alpha - A)}{(\alpha - B)} \cdot \frac{B}{A} = k_f \cdot P_o \cdot t \qquad (2)$$

$$A = \{ C + (C^2 - 4M_o/P_o)^{1/2} \}/2$$

$$B = \{ C - (C^2 - 4M_o/P_o)^{1/2} \}/2$$

$$C = 1 + M_o/P_o + 1/P_o \cdot K$$

$$\alpha = P_c/(P_f + P_c)$$

where M_o, P_o, and K designate the concentration of metal salt, the concentration of cyclic peptide, and the binding constant, respectively.

Substituting the relevant values determined from the measurement of the time-resolved ellipticity into Eq. (2), a good linear relationship was obtained. From the slope of the straight lines, k_f values were obtained, which are tabulated in Table II. The k_f for valinomycin/K^+ complex has been reported to be 2.1×10^9 $M^{-1} \cdot min^{-1}$ in methanol(6). The k_f values obtained with the present cyclic octapeptides are much smaller than that reported for valinomycin. Figure 9(b) shows the time-resolved

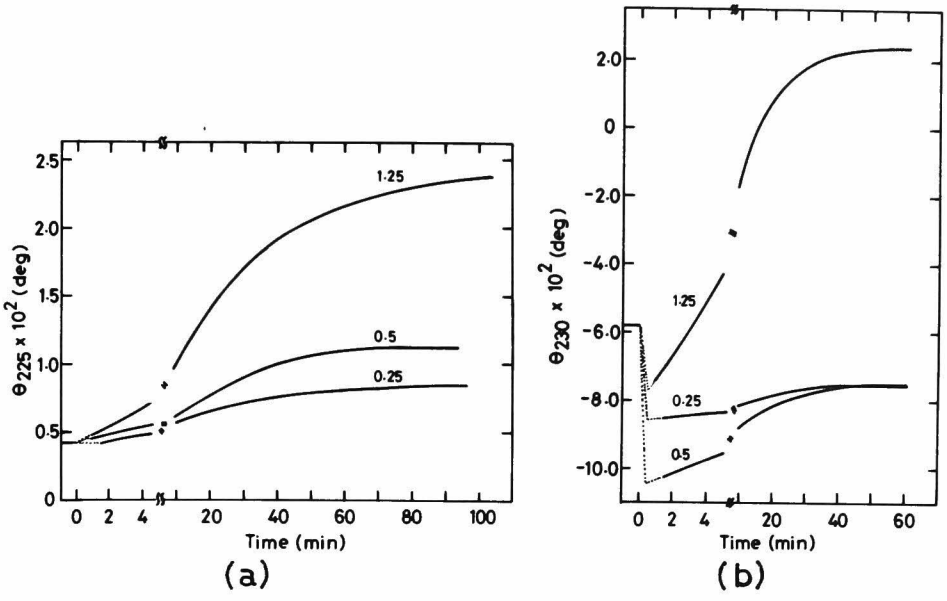

Fig. 9. Time-resolved ellipticity of cd spectra of cyclo(Leu-Pro)$_4$ in the presence of $Ba(ClO_4)_2$. (a) in 95% CH_3OH, (cyclo(Leu-Pro)$_4$)=$5.2\times10^{-4}M^{-1}$; (b) in CH_3CN, (cyclo(Leu-Pro)$_4$)=1.0×10^{-3} M^{-1}. Numbers represent the molar ratio of metal salt added against cyclo(Leu-Pro)$_4$.

TABLE II

Rate Constants of the Complex Formation between Cyclic Octapeptide and Metal Salt ($M^{-1}\cdot min^{-1}$)

	$Ba(ClO_4)_2$	KCl
Cyclo(Leu-Pro)$_4$	0.7 [a]	1.6 [b]
Cyclo(Lys(Z)-Pro)$_4$	12 [c]	n.d.

[a] 95% CH_3OH, 25.0°C [b] 86% CH_3OH, 25.0°C

[c] 95% C_2H_5OH, 24.7°C

ellipticity at 230 nm of cd spectra of cyclo(Leu-Pro)$_4$ in acetonitrile. This pattern of spectral change is completely different from that observed in 95% CH_3OH/H_2O mixture. A rapid decrease of the ellipticity is found immediatly after the addition of metal salt and a gradual increase of the ellipticity followed. Taking the formation of three kinds of species into consideration, the mechanism of the complexation is represented in Figure 10. That is, in acetonitrile the interconversion between a C_2-symmetric conformation and a C_4-symmetric conformation with all-trans peptide bonds is rapid and the complex formation from the latter is also rapid. However, the conversion into the C_4-symmetric conformer with all-cis arrangements of Leu-Pro peptide bonds is as slow as that occurring in methanol. Since the rapid conformational change is accompanied by the isomerization of the peptide bonds, the complex formation of cyclo(Leu-Pro)$_4$ must be much slower than that of valinomycin. The maximum k_f value of the complex formation accompanied by the isomerization of peptide bonds could be $10^{-1}\sim10^{-2}$ sec. However, the change from a C_2-symmetric conformation into a C_4-symmetric one with all-cis arrangements of Leu-Pro peptide bonds was still much slower than the expected value. The slow cis/trans isomerization has been reported on poly(L-proline) (7), and should occur with a certain type of peptide bond involving Pro residue. The reason for the slow conformational change remains to be investigated.

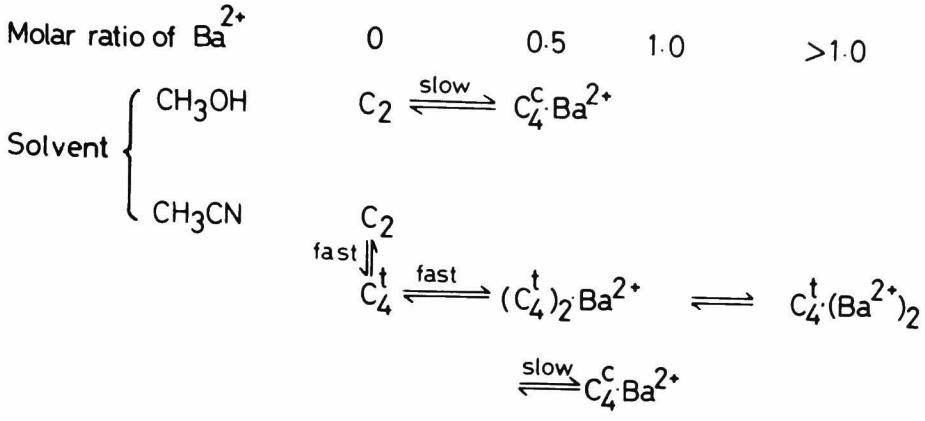

Fig. 10. Complexation of cyclo(Leu-Pro)$_4$ with $Ba(ClO_4)_2$. C_2, C_4^c, and C_4^t represent C_2-symmetric, C_4-symmetric with all-cis arrangements of Leu-Pro peptide bonds, and C_4-symmetric conformer with all-trans peptide bonds, respectively.

To sum up, in a series of cyclic octapeptides investigated here, cyclo(Phe-Pro)$_4$, which has a rigid skeleton, did not form metal-ion complex in alcohol. On the other hand, cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$, which have flexible skeletons, formed complexes with Ba^{2+}, Ca^{2+}, and K^+. The metal-ion selectivity should depend on a delicate balance between the flexibility and the rigidity of ring skeleton.

One of the two kinds of complex formation in acetonitrile was fast, but the other one and that in alcoholic solution were slower than that expected from the usual isomerization rate of peptide bonds. Because the conformation of cyclic octapeptide in the complex is C_4-symmetric, a cyclic octapeptide taking a C_4-symmetric

conformation in a free state will be an efficient ionophore model.

The pattern of complex formation depended strongly on the environment. So, it is necessary to investigate the thermodynamics and kinetics of the complexation between cyclic peptides and metal ions under various conditions to develop effective ionophore model compounds.

Cyclic octapeptides investigated here tends to take C_2-symmetric conformations due to the presence of Pro residues which facilitate the formation of β-turn structure. Cyclo(D-N-methyl-Leu-L-N-methyl-Leu)$_4$ which does not include the Pro residue is thought to take a C_4-symmetric conformation and to show a high lipophilicity for the lack of amide protons. Furthermore, because of a moderate steric repulsion between side chains and N-methyl groups a high ion-selectivity is expected.

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PART III

Membrane Activities of Oligopeptides

Chapter 7

Ion Transport through Liquid Membrane by Synthetic Cyclic Octapeptides

INTRODUCTION

A lot of antibiotics have been found to control the cation permeability through the biomembrane. Some of them are considered to transport cations according to the carrier mechanism. This class of antibiotics possess in the molecule a hydrophilic cavity to bind an ion and a hydrophobic exterior(1). Crown ethers have been shown to take this kind of structure and carry ions by the carrier mechanism(2). However, the factors determining the rate of ion transport through the lipid membrane have not been elucidated. For example, the relation between the rate of ion transport and the stability constant of the complex or the ability of ion extraction from aqueous phase to organic phase differs greatly from an ionophore to others. Macrolide actin, valinomycin, and dicyclohexyl-18-crown-6 are known to transport K^+ ion through the mitochondrial membrane. However, the relative rates of ion transport estimated on the basis of the relative abilities of ion extraction were 1/10 for valinomycin and 1/1000 for dicyclohexyl-18-crown-6, that for macrolide actin being taken as a standard(3). The disagreement has been discussed in terms of the distribution of carrier molecules in the membrane and the ease of the conformational change from a hydrophilic complex formed at the membrane/water interface into a

hydrophobic complex which is suited for permeation through the membrane.

The molecular mechanism governing the ion transport through hydrophilic/hydrophobic cell membranes is complex and is to be investigated further by using suitable model compounds. While many kinds of peptide-antibiotics have been known, only a few studies using synthetic cyclic peptides for the ion transport through the membrane have been reported(4,5). In this chapter, cyclic octapeptides containing proline residues were examined with the ion transport through liquid membrane and the interactions with liposome. In consequence, these cyclic octapeptides were shown to behave differently in the ion transport through liquid membrane and in the rapid response in liposome.

EXPERIMENTAL

Materials

The syntheses and the characterization of cyclic octapeptides, cyclo(Phe-Pro)$_4$, cyclo(Leu-Pro)$_4$, and cyclo(Lys(Z)-Pro)$_4$ have been described previously(6). 18-crown-6 (18-C-6,Aldrich) and valinomycin (Sigma) were used without further purification.

Method

Cation Transport

Figure 1 shows the apparatus used for the cation transport through liquid membrane. The chloroform phase containing ion carriers separates the aqueous phases I

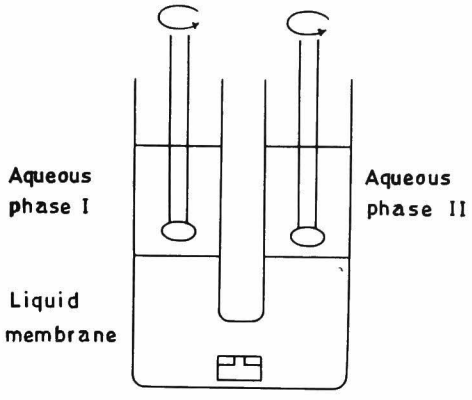

Fig. 1. U-shaped tube for ion transport experiments.

and II, in the former metal chlorides (10mM), picric acid (25mM), and HEPES (10mM, pH 7.2) being contained. The two phases were agitated by HEIDON's three-one motor.

The concentration of metal cations delivered from the aqueous phase I to II was determined by measuring the absorption of accompanying picrate anion. The molar extinction coefficient ε of picrate anion was taken as 1.32×10^4 at 355 nm in aqueous solution and 9.8×10^3 at 420 nm in chloroform(7).

Extraction Procedure

A chloroform solution containing an appropriate cyclic octapeptide was brought into contact with the aqueous solution containing $BaCl_2$ (10mM), picric acid (25mM), and HEPES (10mM, pH 7.3) and agitated for 3 hours at 20°C. After standing for 16 hours, the absorption of picrate anion in chloroform phase was measured at 24°C.

Picrate Transport

The chloroform solution containing cyclo(Leu-Pro)$_4$ (170μM) was agitated with the aqueous solution I containing KCl (0.1M), picric acid (0.6mM), and Tris-H_2SO_4 (10mM, pH 8.2). This procedure was repeated several times with new aqueous solution I, and finally an equilibrium state was reached. After this treatment the aqueous solution I, the equilibrated chloroform solution, and the aqueous phase II containing LiCl (0.1 M), picric acid (0.6mM), and Tris-H_2SO_4 (10mM, pH8.2) were placed in the apparatus of Figure 1 and the absorption change of picrate anion in the aqueous phases I and II were measured.

Preparation of Liposome

Egg yolk lecithin (EYL) was extracted from hen egg and purified according to Singleton(8). Liposome was obtained after the sonication of the EYL dispersion and the ultracentrifugation at 100,000g.

RESULTS and DISCUSSION

Cation Transport

Figure 2 shows the results of cation transport from the aqueous phase I to II by cyclic octapeptides. In the absence of the cyclic octapeptides the concentration of the picrate anion in the aqueous phase II scarcely increased. All of these cyclic octapeptides transported K^+ and Ba^{2+} and cyclo(Leu-Pro)$_4$ was more efficient than cyclo(Phe-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$.

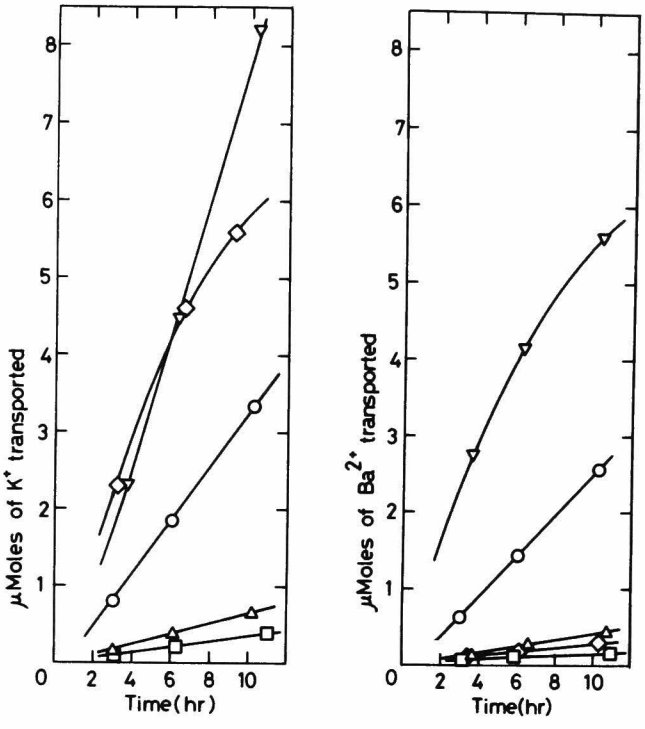

Fig. 2. Transport of cations through chloroform membrane by ▽,18-crown-6(140μM); ◇,valinomycin(140μM); ○,cyclo(Leu-Pro)$_4$(140μM); △,cyclo(Phe-Pro)$_4$(150μM); □,cyclo(Lys(Z)-Pro)$_4$(170μM). Other conditions are described in the experimental section.

Cyclo(Leu-Pro)$_4$ (140μM) transported 3.2 μmoles of K^+ for 10 hours. Deber and Adawadkar reported that cyclic octapeptide cyclo(Glu(OBzl)-Sar-Gly-N-DecylGly)$_2$ (200μM) transported 4.3 μmoles of K^+ for 10 hours(9). Though the quantitative comparison is impossible for different concentrations of metal chloride and picric acid between the two experiments, the abilities of K^+ transport of these cyclic octapeptides seem to be similar.

The cation transports by 18-C-6 and valinomycin are also shown in Figure 2. The micromoles of K^+

transported by 18-C-6, valinomycin, and cyclo(Leu-Pro)$_4$ for 10 hours were 7.9, 5.7, and 3.2 (1.0:0.72:0.41), respectively. Lamb et al. investigated the cation transport by a series of crown ethers, and reported that the relative amount of K^+ transported by dicyclohexyl-18-C-6, 18-C-6, and dibenzo-18-C-6 is 1:0.82:0.30(10). Therefore the ability of cyclo(Leu-Pro)$_4$ to transport K^+ is 1/3 times as large as that of dicyclohexyl-18-C-6, which is the most efficient carrier for K^+.

The equilibrium constants of complex formation of these cyclic octapeptides in alcoholic solution have been described in Chapter 6 (Table I). The binding constants with Ba^{2+} decreases in the order cyclo(Lys(Z)-Pro)$_4$ > cyclo(Leu-Pro)$_4$ > cyclo(Phe-Pro)$_4$,which disagrees with the order of cation transport disclosed in the present investigation; cyclo(Leu-Pro)$_4$ >> cyclo(Phe-Pro)$_4$ > cyclo(Lys(Z)-Pro)$_4$. The binding constants of the cyclic octapeptides with K^+ were not very large, whereas the efficient cation transport through the chloroform liquid membrane by these cyclic octapeptides was observed. Especially cyclo(Leu-Pro)$_4$ transported K^+ ion very efficiently.

The poor Ba^{2+} transport by cyclo(Lys(Z)-Pro)$_4$ could be due to the poor solubility of the complex in chloroform. In fact, cyclo(Leu-Pro)$_4$ forms a soluble complex with Ba^{2+} in acetonitrile, whereas in case of cyclo(Lys(Z)-Pro)$_4$ a precipitation occurred. Since the conformational properties of free cyclo(Leu-Pro)$_4$ in chloroform is similar to those in acetonitrile, the metal-ion complexation of cyclo(Leu-Pro)$_4$ may be similar in both solvents.

So, the solubility of Ba^{2+} complex by cyclo(Lys(Z)-Pro)$_4$ in chloroform was supposed to be poor. On the contrary, the efficient K^+ transport by cyclic octapeptides

could be due to the ease of complex formation with K^+ in chloroform. In fact, the addition of K^+ to cyclo-$(Leu\text{-}Pro)_4$ in acetonitrile induced a drastic change of cd spectrum indicating a complex formation, although the complex formation of cyclo$(Leu\text{-}Pro)_4$ with K^+ was difficult in 86% CH_3OH as shown in Table I. A different mechanism of the complex formation in acetonitrile from that in 95% CH_3OH was suggested from nmr spectra(6).

The cation transport by crown ethers through liquid membrane has been investigated in relation to the stability constants of complex(10). An excellent correspondence between the stability constants and the extraction constants, K_{ex}, was obtained with macrolide actins(11). The extraction of barium pictate ($BaPi_2$) from aqueous phase to chloroform phase by these cyclic octapeptides was also examined (Table I). Assuming the formation of 1:1 complex between Ba^{2+} and cyclic peptides, K_{ex} was calculated according to Eq. (1). Constant K_{ex} was

$$K_{ex} = \frac{(BaPi_2 \cdot Peptide)_{CHCl_3}}{(Ba^{2+})_{H_2O} \cdot (Pi)^2_{H_2O} \cdot (Peptide)_{CHCl_3}} \quad (1)$$

obtained which was independent of the concentration of the cyclic peptide. The ratio of the amount of ion transported against K_{ex} was almost the same among these cyclic octapeptides. This indicates that the ion transport by the cyclic peptides through liquid membrane is determined by their ability of Ba^{2+} extraction into chloroform phase.

Figure 3 shows the ion selectivity of cyclo$(Leu\text{-}Pro)_4$ observed in the cation transport through chloroform membrane. The rate of alkali cation transport increases

TABLE I

Extraction Consatants and Equilibrium Constants of Cyclic Octapeptides

	(Peptide) in $CHCl_3$, μM	Extraction constants, M^{-3}	μMoles of Ba^{2+} transported (A)	A/K_{ex}
cyclo(Leu-Pro)$_4$	50	6.8×10^4	2.3	3.0×10^{-5}
	100	7.6×10^4		
	150	8.0×10^4		
		av. 7.5×10^4		
cyclo(Phe-Pro)$_4$	50	1.1×10^4	0.28	3.0×10^{-5}
	100	0.90×10^4		
	150	0.85×10^4		
		av. 9.5×10^3		
cyclo(Lys(Z)-Pro)$_4$	100	0.44×10^4	0.12	2.6×10^{-5}
	150	0.47×10^4		
		av. 4.5×10^3		

	cyclo(Leu-Pro)$_4$	cyclo(Phe-Pro)$_4$	cyclo(Lys(Z)-Pro)$_4$
Binding constant (M^{-1}) for Ba^{2+}	4.2×10^2 [a]	very low	1.3×10^3 [b]
K^+	~ 3 [c]	-	~ 3 [c]

[a] 95% CH_3OH, [b] 95% C_2H_5OH, [c] 86% CH_3OH

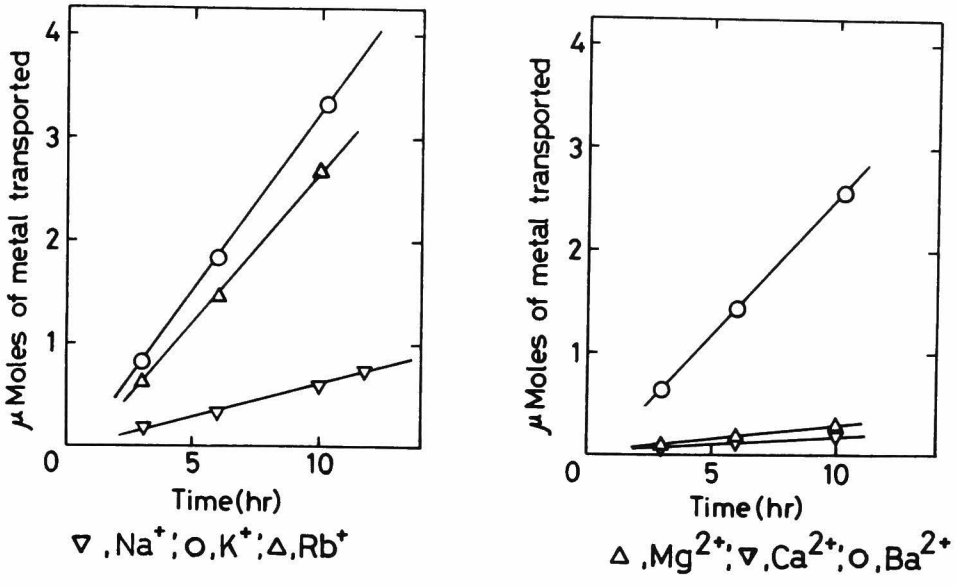

Fig. 3. Transport of various cations by cyclo-(Leu-Pro)$_4$ (140μM) through chloroform membrane. The salt concentrations are described in the experimental section.

in the order $Na^+ < Rb^+ < K^+$. With reference to alkaline-earth cations Ba^{2+} was transported selectively. The relation between the rate of cation transport and the ionic diameters is shown in Figure 4. Thus cyclo(Leu-Pro)$_4$ was shown to transport selectively the cation having a diameter about 2.7 Å. With these cyclic octa-peptides a good correspondence between cd spectra and their conformations has been described in Chapter 6. The conformation of cyclo(Leu-Pro)$_4$/K^+ complex in aceto-nitrile must be a C_4-symmetric all-trans one, because its cd spectrum showed a strong negative n-π* Cotton effect in acetonitrile. Figure 5 shows the Corey-Pauling-Koltun (CPK) molecular model of this conformation.

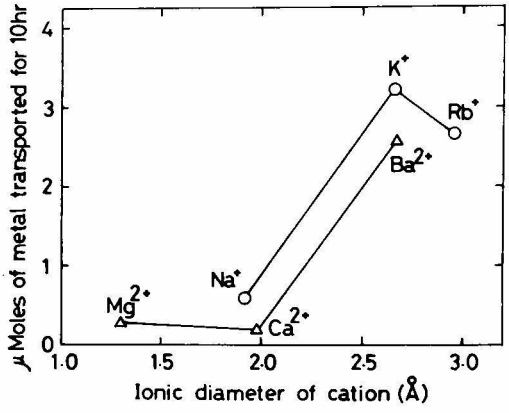

Fig. 4. μMoles of metal ions transported for 10 hours by cyclo(Leu-Pro)$_4$ (140μM) against the ionic diameter of cations.

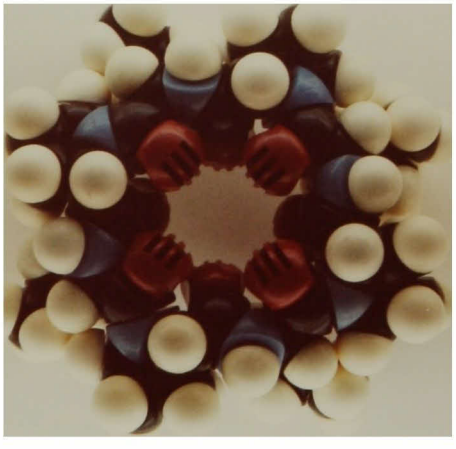

Fig. 5. CPK model of cyclo(Leu-Pro)$_4$ taking an all-trans C_4-symmetric conformation. The black bar shows a width 2.7 Å.

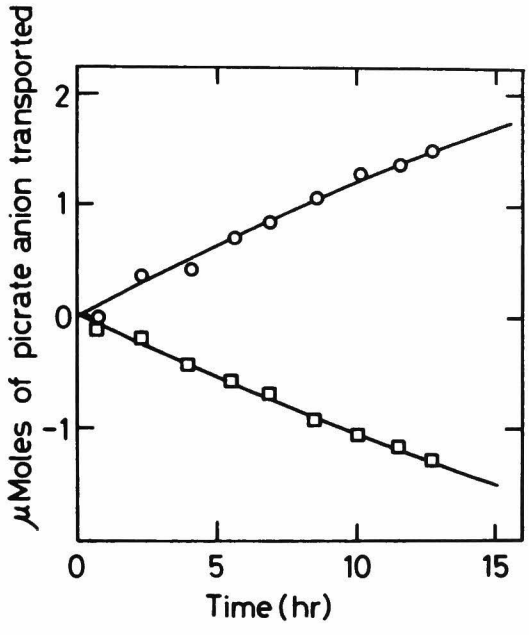

Fig. 6. Change of picrate ion concentration in the aqueous phases I (□) and II (○)induced by the migration of K^+ complex of cyclo(Leu-Pro)$_4$ (170μM). Other conditions are described in the experimental section.

Four carbonyl groups form a hydrophilic cavity which is suitable to bind cations having a diameter about 2.7 Å. Since the structure of the complex in chloroform is reasonably taken as the same as that in acetonitrile, the ion selectivity observed here should be determined by the fit of the cavity formed by carbonyl groups of the cyclic peptide with cations having a specific ionic diameter.

Transport of Picrate Anion against Its Concentration Gradient

To the aqueous solutions I and II containing the same amount of picrate anion was added exactly the same amount of KCl and LiCl, respectively, and the change of

the picrate concentrations in both aqueous phases were measured(12). In the presence of cyclo(Leu-Pro)$_4$ in the middle chloroform phase, picrate anions were transported from the aqueous phase I to II as shown in Figure 6. This phenomenon can be interpreted as follows: cyclo-(Leu-Pro)$_4$ formed a complex selectively with K^+, which was transported from the aqueous phase I to II according to its concentration gradient, while picrate anions were cotransported as counter ions to the aqueous phase II against the concentration gradient. It was therefore proven that cyclo(Leu-Pro)$_4$ works as an ion-selective carrier in liquid membrane.

Cyclo(Leu-Pro)$_4$ in Liposome

Cyclo(Leu-Pro)$_4$ is scarcely soluble in water but soluble in organic solvents except n-hexane and ether, etc. From the dialysis experiment of the liposome solution containing cyclo(Leu-Pro)$_4$, most of cyclo(Leu-Pro)$_4$ added were shown to be bound to the membrane. The cd spectra of cyclo(Leu-Pro)$_4$ in different environments are shown in Figure 7. Changing solvent from H_2O/CH_3OH (1/1 v/v) to ethanol/n-hexane (1/1 v/v) the positive n-π^* Cotton effect ($\sim$230 nm) shifted to longer wavelength as expected. Since the wavelength of this Cotton effect in liposome is 228 nm, cyclo(Leu-Pro)$_4$ is supposed to be located in a polar environment of liposome, which is similar to a 50% aqueous methanol. This region could be a membrane/solvent interface.

Next, the complexation of cyclo(Leu-Pro)$_4$ in liposome was investigated. Figure 8 shows the change of the intensity of cd band at 224 nm of cyclo(Leu-Pro)$_4$ with the change of Ba^{2+} concentration in the exterior of

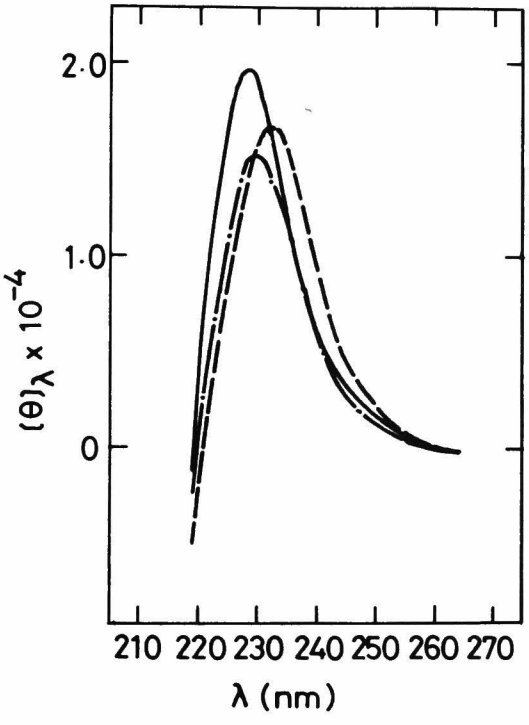

Fig. 7. Cd spectra of cyclo(Leu-Pro)$_4$ in ethanol/n-hexane(1/1 v/v) (———), H_2O/CH_3OH(1/1 v/v) (—·—), and liposome (——) {0.1M NaCl, 0.01M phosphate buffer, pH 7.0, (EYL) = 5 mM, (cyclo(Leu-Pro)$_4$) = 3.3×10^{-4} M}.

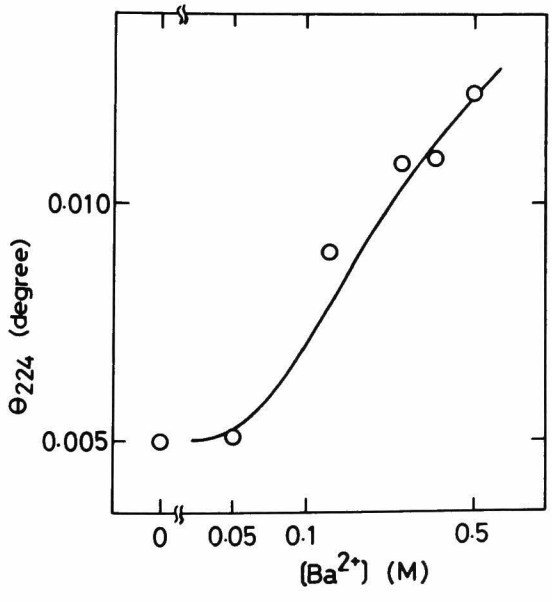

Fig. 8. Change of the ellipticity at 224 nm of cyclo(Leu-Pro)$_4$ in liposome with the change of barium ion concentration. Total salt concentration was kept constant by the compensation by magnesium salt.

liposome. A change was observed when ca. 0.1 M Ba^{2+} was added. So, the equilibrium constant of complex formation in liposome should be lower by more than two orders of magnitude than that in 95% methanol. The reasons should be as follows: i) the complexation at the membrane surface is severely restricted by a strong solvation of ions by water, ii) the effective concentration of cation is diminished at the membrane interface, and/or iii) the transformation of the conformation of complex into one suitable to permeate through the membrane is difficult. A low binding constant in liposome is also observed with naturally-occurring cyclic depsipeptide enniatin B(13).

When the concentration gradient of cation across the membrane exists and a carrier for the cation is present in the membrane, the membrane potential according to the Nernst equation should be formed. Using cyclo(Leu-Pro)$_4$ as a carrier, the membrane potential in liposome was investigated by the change of the fluorescence of cyanine dye 3,3'-dipropylthiodicarbocyanine iodide (diS-C_3-(5)) (14). However, the membrane potential was not detectable, probably because Cl^- from added electrolyte and other ions migrated rapidly across the membrane to cancel the membrane potential once generated in liposome. On the other hand, the complex formation by cyclo(Leu-Pro)$_4$ was very slow because the latter realizes a necessary conformational change only after the cis/trans isomerization of peptide bonds, which was slower than that expected for usual peptide bonds in this case(6). Therefore, the absence of the membrane potential by cyclo(Leu-Pro)$_4$ is supposed to be due to the slow complex formation. The slow conformational change has also been reported with polyproline(15). Therefore, much attention should

be paid to the rate of conformational change of ion-carrier, when proline-containing peptides are investegated as ionophore models.

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Chapter 8

Interactions of Peptides in the Assembly of Lipid Molecules by Fluorescence Behavior

INTRODUCTION

The investigation on the protein-protein and protein-lipid interactions in a membrane is a key to elucidate the mechanism of membrane functions. For instance, several studies have been devoted to examine whether the membrane proteins diffuse freely in a membrane(1,2) or they form clusters(3). In these investigations artificial membranes reconstituted from membrane proteins extracted from biomembranes and lipid molecules were used instead of intact biomembranes. Random collisions caused by the lateral diffusion in the membrane of phosphatidylcholine vesicles have been shown between NADPH-cytochrome P-450 reductase and cytochrome P-450, which are the components of the electron transport system in liver microsomes(4). A stoichiometric complex formation among various subunits has been shown in the reconstruction of the H^+-ATPase-membrane(5). On the other hand, water-soluble polypeptides (6-8) and oligopeptides(9) have been used to study the interaction with liposome. These investigations showed the evidence for the electrostatic interaction between polar head groups of lipids and the peptides.

In this chapter, the distribution of hydrophobic oligopeptides in the hydrophobic region of artificial membranes and the peptide-peptide and the peptide-lipid

interactions in the membranes were investigated. Blout et al. observed by cd, nmr and ir spectroscopy a dimer formation of hydrophobic oligopeptides by hydrogen bonding in artificial membranes(10). In the present investigation fluorescent probes were introduced to peptides and lipids and their interactions in artificial membranes were investigated through the fluorescence behavior and the energy transfer between the probes. Generally speaking, the fluorescence sensitively reflects the environment of the probe, and the energy-transfer efficiency can be related to the distance and the orientation between the probes. The present fluorescent measurements aimed at the clarification of the nature of peptide-lipid and peptide-peptide interactions in membranes and the effect of the phase transition of membrane on the distribution of the probes.

EXPERIMENTAL

Synthesis of Fluorescent Probes

Boc-Try-OH was condensed with appropriate amino acid ester hydrochloride by DCCI to obtain Boc-Try-Phe-OEt, Boc-Try-Leu-OEt, Boc-Try-Val-OEt, Boc-Try-Pro-OMe, and Boc-Try-Gly-OEt. Boc-Lys(Z)-Phe-OEt was synthesized in a similar way, the benzyloxycarbonyl group was removed by catalytic hydrogenation, and the condensation with anthracene-9-carboxylic acid was carried out by DCCI/HOBt to obtain Boc-Lys(Anth)-Phe-OEt, thus an anthryl group being introduced to the side chain of a Lys residue. 12-(9-anthroyloxy)stearic acid (12-AS) was synthesized according to Lennard(11). All materials synthesized were identified by ir and elementary analysis. 2-(9-

anthroyloxy)stearic acid (2-AS) was purchased from Molecular Probes Inc.

Preparation of Liposome

Egg yolk lecithin (EYL) was extracted from hen egg and purified according to Singleton(12). Dipalmitoylphosphatidylcholine was purchased from Fluka AG. Liposome was prepared by sonication of the dispersion of lipids in a phosphate-buffered aqueous medium (0.1 M NaCl, 10 mM phosphate, pH 7.05) and ultracentrifugation at 100,000g. An aliquot of the ethanolic solution of fluorescent probe was added to the liposome.

Measurement

The fluorescence spectra were obtained by the excitation at 281 nm. The apparent energy-transfer efficiency (T_{app}) was calculated from the intensity at 290 nm of excitation spectrum. The excitation spectra were obtained by monitoring the fluorescence of anthryl group (12-AS and 2-AS, 460 nm; anthracene, 425 nm; Boc-Lys(Anth)-Phe-OEt, 415 nm). The 100% transfer and the 0% transfer were determined from the sum of absorption spectra of energy donors and energy acceptors and from absorption spectra of acceptors, respectively. The excitation and the absorption spectra were normalized with the intensity at the maximum absorption wavelength of acceptors(13). The fluorescence bands were overlapped between the donor and the acceptor to some extent. Therefore, the true efficiency of energy transfer (T) was obtained after the correction of T_{app} by Eq. (1) was made.

$$T_{app} = T + \left(\frac{\Phi_{Try} \cdot F(Try)}{\Phi_{Anth} \cdot F(Anth)} \right) \cdot (1 - T) \qquad (1)$$

Φ_{Try} and Φ_{Anth} mean the quantum yields of the donor and the acceptor, respectively. F(Try) and F(Anth) mean the ratios of the fluorescence intensity at the monitoring wavelength against the area of the fluorescence spectra of the donor and the acceptor, respectively. The quantum yield was measured with reference to 9, 10- diphenylanthracene(14).

RESULTS AND DISCUSSION

Solubility of Dipeptides to Liposome

Linear dipeptides containing tryptophan were synthesized as fluorescent peptides, and their N-terminal and C-terminal were blocked by Boc group and ester group, respectively, to provide the peptides with the solubility in membrane. The addition of EYL liposome to these linear dipeptides resulted in a blue shift of the maximum wavelength of fluorescence (λ^{em}_{max}) of the Try residue (Figure 1). Since λ^{em}_{max} shifts to shorter wavelength by the decrease of solvent polarity(15), the observed blue shift indicates the solubilization of the linear peptides into the hydrophobic region of liposome. The degree of the shift depended on the nature of the other residue than Try in the dipeptides. It decreased in the order Phe > Leu > Val > Pro > Gly. This order agrees well with that of the hydrophobicity scale of amino acids (16), and the more hydrophobic linear dipeptide is buried

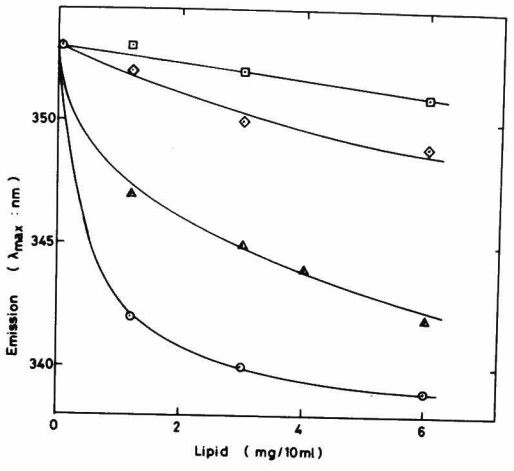


Fig. 1. Change of the maximum wavelength of fluorescent linear dipeptides in EYL liposome. ○, Boc-Try-Phe-OEt; △, Boc-Try-Val-OEt; ◇, Boc-Try-Pro-OMe; □, Boc-Try-Gly-OEt. (Linear dipeptide) = 7×10^{-6} M.

more deeply into the membrane. In the same experiments with cyclic dipeptides synthesized from the above-mentioned linear dipeptides, a similar tendency was observed. However, the degree of blue shift was smaller than that of linear dipeptides, indicating that the terminal Boc and ester groups raise the hydrophobicity of peptide.

The partition coefficients of linear dipeptides to the membrane were analyzed by dialysis experiments. The change of fluorescence intensity before and after the dialysis of peptide/liposome mixture was measured. The probe number in the water phase against that in the membrane was calculated to be 3.6×10^{-3} for Boc-Try-Phe-OEt and 5.9×10^{-2} for Boc-Try-Gly-OEt. These values

indicate that almost all peptide molecules are partitioned in the membrane. The small λ_{max}^{em} shift of Boc-Try-Gly-OEt may be due to the solubilization to the membrane surface.

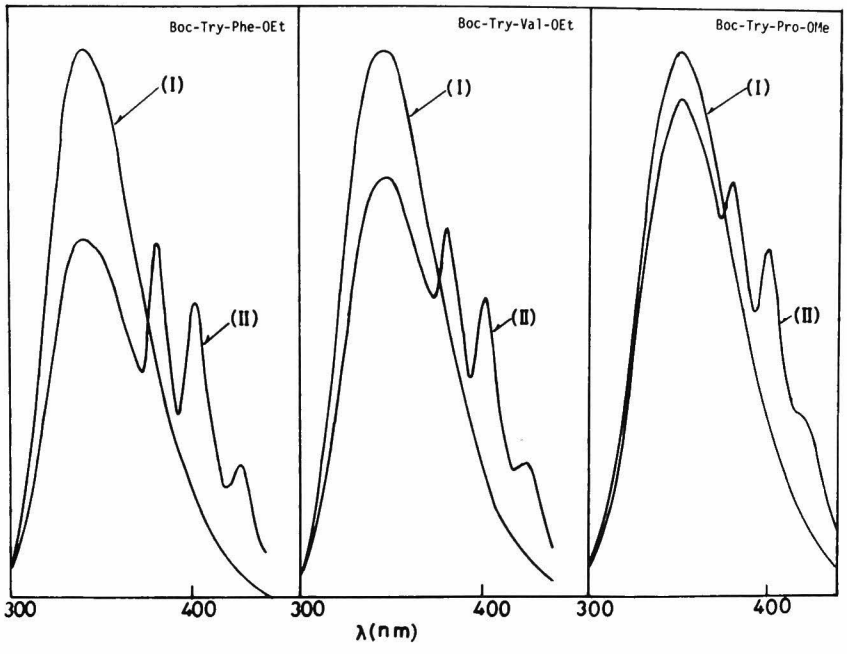
Energy Transfer from Dipeptides to Anthracene in Liposome

The addition of anthracene to the peptide/EYL liposome resulted in the decrease of fluorescence intensity of Try residue, while the fluorescence of anthracene newly appeared. This indicates the occurrence of energy transfer from excited indolyl group to anthracene (Figure 2(a)).

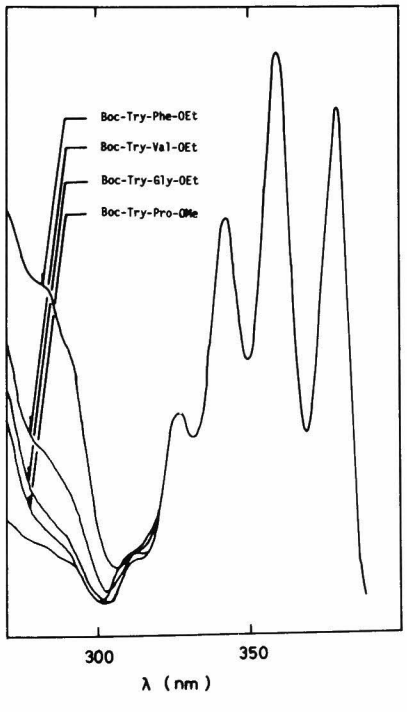
The energy-transfer efficiency, calculated from the excitation spectra (Figure 2(b)), decreased in the order Boc-Try-Phe-OEt(36%) > Boc-Try-Val-OEt(14%) > Boc-Try-Gly-OEt(3%) ≃ Boc-Try-Pro-OMe(2%). This order agrees well with the ease for the peptides to dissolve into the hydrophobic region of membrane as stated above. Under the same conditions but in ethanol, the energy transfer could hardly be observed. Therefore, the energy-transfer efficiency should increase as the probe concentration in the hydrophobic region of membrane increases.

Energy Transfer in DPPC Liposome

A phase transition from a gel to a liquid crystalline of DPPC liposome occurs at 42°C(16), and at lower temperatures the mobility of fluorescent probe in the membrane should be restricted significantly. The effect of phase transition of the membrane on the interactions between the probes was investigated.



(a)



(b)

Fig. 2. (a) Emission spectra in the absence (I) or the presence (II) of anthracene and (b) excitation spectra in the presence of anthracene in EYL liposome. (Donor) = 7×10^{-6} M, (Anthracene) = 0 or 3.5×10^{-6} M.

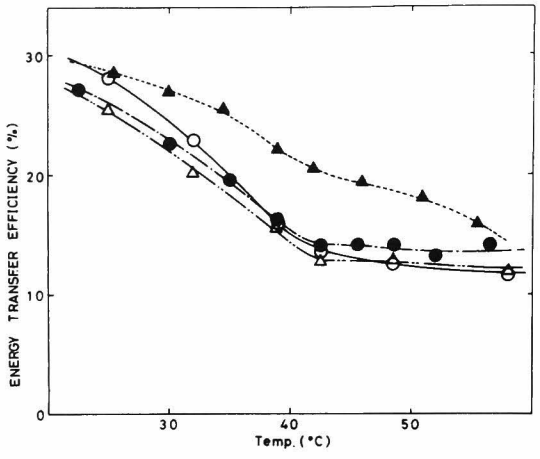


Fig. 3. Temperature dependence of the energy-transfer efficiency in DPPC liposome from Boc-Try-Phe-OEt to different acceptors. ○, 12-AS; ●, 2-AS; △, anthracene; ▲, Boc-Lys(Anth)-Phe-OEt. (Donor) = (Acceptors) = 3.3 x 10^{-6} M.

Figure 3 shows the temperature dependence of the energy-transfer efficiency from Boc-Try-Phe-OEt to various acceptor molecules. The temperature dependence of the efficiency of energy transfer to lipid acceptors and anthracene showed a bend around the phase-transition temperature. The energy-transfer efficiencies were almost constant at higher temperatures than the phase-transition temperature. On the other hand, it increased as the temperature falls at lower temperatures than the phase-transition temperature. The latter indicates that the phase transition of membrane strongly influences the interaction between the probes in the membrane. In the

case of the energy transfer from Boc-Try-Phe-OEt to Boc-Lys(Anth)-Phe-OEt the bend in the temperature dependence curve was gentle. The energy-transfer efficiency was larger than those to other acceptors, and it increased as the temperature falls at lower temperatures than the phase-transition temperature. The anthryl group of 2-AS is located in the surface region of membrane, while that of 12-AS is located deeply in the membrane. In spite of the difference between them, no great difference of the energy-transfer efficiency was found between them. On the other hand, a different behavior was observed in the case of peptide acceptors as stated above. Therefore, the nature of the peptide-lipid and peptide-peptide interactions must be different. In other words, a loose complex might be formed by hydrogen bonding among peptides in the membrane as pointed out by Blout et al.(10).

Since the motions of the probes in the membrane are restricted at lower temperatures, the increase of the energy-transfer efficiency by the temperature fall cannot be explained in terms of the mobility of probe. Eq. (2) relates T, k_{tr}, k_f, and Φ_{Try} with each other, which represent the energy-transfer efficiency, the rate of energy transfer, the probability of radiative transition and the quantum yield of donor, respectively.

$$\ln \left(\frac{k_{tr} \cdot (\text{Acceptor})}{k_f}\right) = \ln \left(\frac{T}{1 - T} \cdot \frac{1}{\Phi_{Try}}\right) \quad (2)$$

Substituting T and Φ_{Try} observed at appropriate temperatures for Eq. (2), the logarithms of $k_{tr} \cdot (\text{Acceptor})/k_f$ were obtained and are plotted against 1/T in Figure 4, $\ln(k_{tr} \cdot (\text{Acceptor})/k_f)$ increased with lowering the temperature. The temperature dependence of Φ_{Try} in liposome

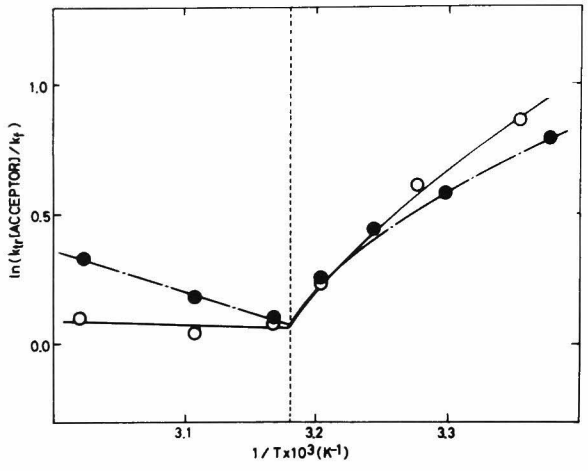


Fig. 4. Arrhenius plot of $k_{tr}\cdot(\text{Acceptor})/k_f$. Donor: Boc-Try-Phe-OEt. Acceptor: ○, 12-AS; ●, 2-AS.

was found to increase slightly as the temperature fell, but it did not show a bend around the phase-transition temperature. This indicates that k_f does not change drastically by the phase transition. Therefore, the increase of the energy-transfer efficiency at lower temperatures is ascribable to either the increase of k_{tr}, which is related to the critical energy transfer distance (R_o), or the increase of the local concentration of acceptor in the neighborhood of donor.

Dependence of the Energy-Transfer Efficiency on the Probe Concentration

The temperature dependence of the energy transfer from Boc-Try-Phe-OEt to 12-AS was determined under various concentrations of the probe, and is plotted in Figure 5. The efficiency was independent of the concentration of donor but dependent on the acceptor concentration within the temperature range studied. This means a random distribution of the peptide donor and the lipid acceptor in the membrane. Under these conditions , the dependence of the energy-transfer efficiency on the acceptor concentration was calculated according to Fung's equation(18),

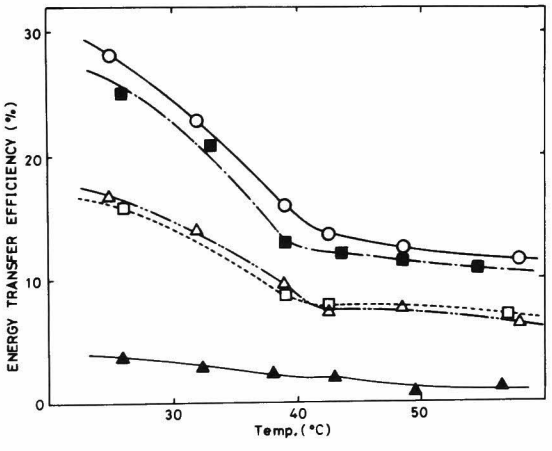


Fig. 5. Temperature dependence of the energy-transfer efficiency under various probe concentrations. (Boc-Try-Phe-OEt)/(12-AS): ○ , 3.3 x 10^{-6}M/3.3 x 10^{-6}M; ■ , 1.65 x 10^{-6}M/3.3 x 10^{-6}M; □ , 3.3 x 10^{-6}M/1.65 x 10^{-6}M; △ , 1.65 x 10^{-6}M/1.65 x 10^{-6}M; ▲ , 3.3 x 10^{-7}M/3.3 x 10^{-7}M.

and R_o was calculated according to Förster's equation(19). The orientation factor κ^2 was taken as 2/3 at higher temperatures, where a random orientation is assumed. At lower temperatures, the orientation of the donor and the acceptor in a crystalline lipid bilayer may be fixed in a favorable way for the energy transfer. Table I shows the result of the fluorescence depolarization experiment. In ethanol solution the depolarization of fluorescence was almost complete. On the other hand, in liposome the fluorescence is highly polarized, which indicates a low mobility of the probe in the membrane. In DPPC liposome at 25°C which is lower than the phase-transition temperature, the degree of polarization was as high as 0.1∿0.2. According to Haas et al.(20) κ^2 takes a value of 1.436, when the polarizations of donor and acceptor are 0.1 and 0.2, respectively and their orientation is most favorable for the energy transfer. Using 2/3 at 55°C and the maximum value 1.436 at 25°C for κ^2, R_os were calculated to be 22.5 Å and 27.2 Å, respectively. The energy-transfer efficiencies based on these values of R_o are shown in Figure 6. In Figure 6, the efficiencies observed at 55°C and 25°C are also plotted. The surface density of the acceptor was calculated assuming their random distribution at outer surface of liposome. Figure 6 shows that the observed values at 55°C agree well with the theoretical calculation, while the observed values at 25°C are definitely larger than the theoretical values which is based upon the maximum value of R_o. Therefore, the increase of energy-transfer efficiency at lower temperature cannot be explained merely by the increase of R_o. It may be explained in terms of an increased surface density of acceptor in the neighborhood of a donor molecule.

TABLE I

Polarization of Various Fluorescent Probes under Different Circumstances

Probe	Fluorescence Polarization			
	Ethanol	Liposome		
		EYL	DPPC(25°C)	DPPC(55°C)
Anthracene	0.010	0.189	-	-
2-AS	0.030	0.120	-	-
12-AS	0.020	0.045	0.143	0.045
Boc-Lys(Anth)-Phe-OEt	0.030	0.182	0.195	0.140
Boc-Try-Phe-OEt	-	-	0.095	0.050

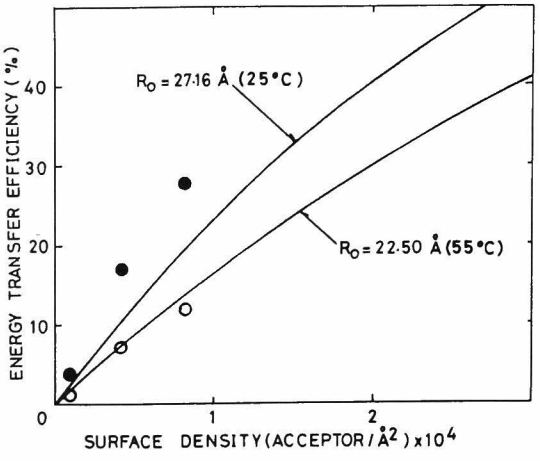


Fig. 6. Dependence on the surface density of the energy-transfer efficiency. The curves represent the theoretical values calculated by Fung's equation for each R_o value indicated. O (55°C) and ● (25°C) are observed values (see Figure 5). Donor: Boc-Try-Phe-OEt, Acceptor: 12-AS.

The local increase of the surface density of acceptor might be explained as follows. At higher temperatures the probes distribute randomly over the surface of vesicle. However, under phase-transition conditions lipid molecules begin to crystallize to induce a phase separation in the membrane. Consequently the domains containing high concentrations of the probes are formed. Since the probes are randomly distributed in the domains, the energy-transfer efficiency does not depend on the concentration of the donor but on the acceptor concentration. McGrath et al. (21) reported from the photo-bleaching experiment that under phase-

transition conditions 12-AS is excluded from the DPPC gel-matrix and forms a condensed region, which agrees with the present experimental results.

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Chapter 9

Intramolecular Transfer of Excitation Energy, Complexation with Metal Ions, and Interactions with Liposome of Cyclic Pentapeptide Containing a Tryptophan Residue

INTRODUCTION

As described in Chapters 2 and 8, the fluorescence measurement is an useful method for the investigations of peptides in lipid membrane. In this chapter, The complexation with metal ions and the fluorescent behaviors of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in organic solvents or in lipid membrane were investigated. In this cyclic pentapeptide a Try residue, an energy donor group, and a 5-dimethylaminonaphthalene sulfonyl group, an energy acceptor group, exist within the same molecule. The intramolecular transfer of excitation energy occurring in the cyclic peptide was also investigated in ethanol and DPPC liposome.

In multifunctional molecules the cooperation of elementary functions is expected. For example, a cyclic hexapeptide, cyclo(D-Leu-Glu-His)$_2$, was an asymmetric hydrolytic catalyst of leucine or valine p-nitrophenyl ester hydrochlorides only when it formed a complex with Cu^{2+}(1). Bis-crown ethers, in which the two crown ether moieties are covalently bound by an azobenzene group, realized a photoregulated cooperative complex formation with metal ions through the cis/trans

isomerization of the azobenzene group(2). The cyclic pentapeptide, cyclo(Try-Sar-Sar-Lys(DNS)-Pro), is provided with two functions; one is the selective complex formation with metal ions and the other is the energy acceptance and its intramolecular transfer. It should be interesting to know how the intramolecular energy transfer is affected by the complexation with metal ions. This was really investigated in 95% methanol solution.

EXPERIMENTAL

Materials

Syntheses of cyclic pentapeptides investigated here have already been described in Chapter 2.

DPPC was purchased from Fluka AG. 2-(9-anthroyloxy)---stearic acid (2-AS) was purchased from Molecular Probes Inc.

Preparation of Liposome

The lipid was dispersed in 10 mM phosphate buffered solution (pH 7.1) containing 0.1 M NaCl, and was sonicated and ultracentrifuged at 100,000g to obtain liposome. The ethanol solution of fluorescent probe was added to liposome.

Measurements

Fluorescence spectra were obtained by the excitation at 281 nm. Fluorescence excitation spectra were obtained by monitoring the fluorescence from the dansyl group at 520 nm. 100% energy transfer was determined from

the absorption spectrum of cyclo(Try-Sar-Sar-Lys(DNS)-Pro). 0% transfer was estimated from the excitation spectrum of cyclo(Try(CHO)-Sar-Sar-Lys(DNS)-Pro), because the N^{i}-formyltryptophan scarcely fluoresces. Both absorption and excitation spectra were normalized with the absorption intensity at the wavelength of the maximum absorption of dansyl group. The energy-transfer efficiencies were calculated from the intensity at 290 nm of Try in the excitation spectra of cyclo(Try-Sar-Sar-Lys(DNS)-Pro).

RESULTS AND DISCUSSION

Complexation of Cyclo(Try-Sar-Sar-Lys(Z)-Pro) with Metal Cations

Complexation of cyclic peptides with metal ions has been correlated with the flexibility of ring structure(3). Cyclo(Try-Sar-Sar-Lys(Z)-Pro) contains three N-substituted peptide bonds, which make cis as well as trans configuration possible. Therefore, this cyclic pentapeptide is expected to bind metal ions efficiently due to a number of available conformations. On the other hand, the ring size is not large enough to provide the cyclic pentapeptide with a sufficient flexibility for the complexation.

With the addition of KCl or $NaClO_4$ by 10 or 20 times as much as cyclo(Try-Sar-Sar-Lys(Z)-Pro) in 95% aqueous methanol solution, no change of cd spectrum was observed, giving no proof of complexation. On the other hand, with the addition of $Ba(ClO_4)_2$, the change in cd spectra was observed as shown in Figure 1, indicating the complex formation. From the variation

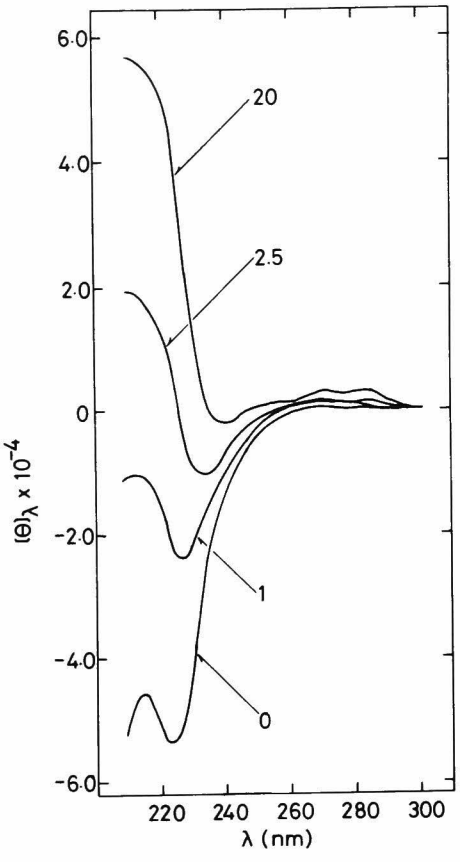

Fig. 1. Change of cd spectra of cyclo(Try-Sar-Sar-Lys(Z)-Pro) in CH_3OH/H_2O (95/5 v/v) with the addition of $Ba(ClO_4)_2$. The numbers represent the ratio of $(Ba^{2+})/(\text{cyclic peptide})$.

of cd spectra, the stoichiometric composition of the complex was calculated to be 1:1 and the equilibrium constant of the complex formation 2.0×10^3 M^{-1} (4).

Addition of $Ca(ClO_4)_2$ also caused a similar change in cd spectra, but the equilibrium constant was as small as 10 M^{-1}. Therefore, cyclo(Try-Sar-Sar-Lys(Z)-Pro) forms a complex selectively with Ba^{2+}.

In acetonitrile precipitation occurred with the addition of $Ba(ClO_4)_2$. ^{13}C nmr spectrum of the white precipitation in CD_3OD showed an evidence for the intermixing of different conformations. However, each conformation was unable to be identified.

Intramolecular Excited Energy Transfer of Cyclo(Try-Sar-Sar-Lys(DNS)-Pro)

The temperature dependence of the intramolecular energy-transfer efficiency of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in ethanol is shown in Figure 2. The efficiency of the energy transfer is about 82% between 0 and 40°C. A slight increase of the transfer efficiency was observed at lower temperatures. Since the absorption spectra of this cyclic pentapeptide hardly changed by the temperature, the increase of the transfer efficiency at lower temperatures may be due to the increase of the quantum yield of Try residue.

The critical distance for the energy transfer of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in ethanol was estimated to be 21 Å from the fluorescence spectrum of cyclo(Try-Sar-Sar-Lys(Z)-Pro) and the excitation spectrum of cyclo-(Try(CHO)-Sar-Sar-Lys(DNS)-Pro) (5). Assuming that the conformation of the ring structure of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) is the same as that of cyclo(Try-Sar-Sar-Lys(Z)-Pro) shown in Figure 4 (d)-2 in Chapter 2, the distance between the indolyl and the dansyl groups ranges from 0 to about 20 Å. This large variation is arisen from the flexibility of the Lys side chain. Though both chromophores situate within the critical distance for the energy transfer, the efficiency of the energy transfer was always lower than 100%. The possible reason for this

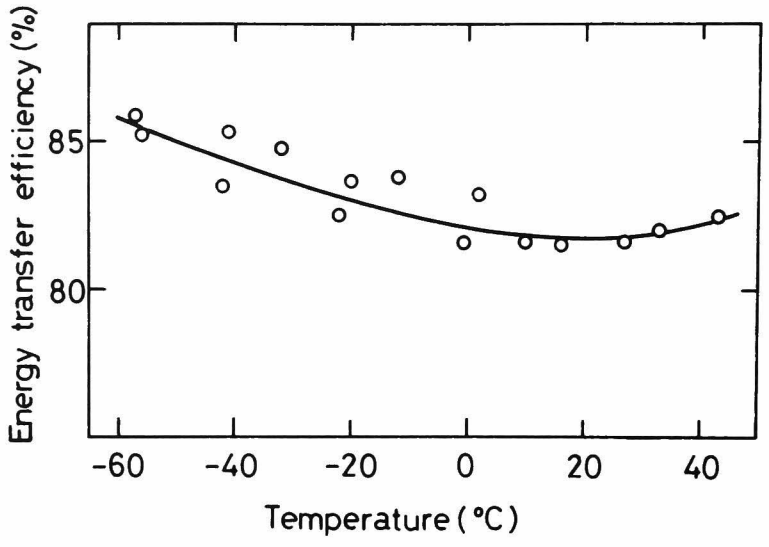

Fig. 2. The temperature dependence of intramolecular energy transfer of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in ethanol. (cyclic peptide) = 1.0×10^{-5} M.

phenomenon could be that two chromophores do not always take the suitable orientation for energy transfer during the life time of the excited indolyl group.

Interaction of Cyclo(Try-Sar-Sar-Lys(DNS)-Pro) with DPPC Liposome

The maximum fluorescence of the Try-indolyl group of cyclo(Try-Sar-Sar-Lys(Z)-Pro) was observed at 340 nm in the presence of DPPC liposome. The maximum fluorescence of indolyl group in aqueous solution usually appears at 350 nm. Therefore, the blue shift of the fluorescence

indicates that the cyclic peptide was buried into the hydrophobic region of DPPC liposome(6). The excitation of cyclo(Try-Sar-Sar-Lys(Z)-Pro) added to DPPC liposome containing 2-AS induced the energy transfer from the cyclic peptide to 2-AS. This event also verifies that the cyclic peptide is bound to the membrane. The efficiency of the energy transfer (under the condition that cyclo(Try-Sar-Sar-Lys(Z)-Pro) , 4.7×10^{-6} M; 2-AS , 3.6×10^{-6} M; DPPC , $\sim$1 mM) was about 4% over the temperature range for the liquid crystalline state of the membrane, while it increased to 11% in the phase-transition region where the membrane is in the crystalline state. This phenomenon must have been caused by an apparent increase of the surface density of the acceptor. The phase separation at the phase-transition temperature produces crystalline regions without the probes and other domains containing high concentrations of probes(7).

The temperature dependence of the intramolecular energy-transfer efficiency of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in DPPC liposome is shown in Figure 3. There is a bend around 34°C, and thereafter the efficiency of energy transfer increased with the temperature fall. Since the intramolecular energy transfer in ethanol was almost constant above 0°C, the increase below 34°C must have been caused by the interaction of the cyclic peptide with DPPC liposome. In DPPC liposome phase transitions occur at 42°C and 34°C. The former is related with packing of alkyl groups of the lipid molecules and the latter with mobility of methyl groups of the polar region of lipid molecules(8). Therefore it is likely that cyclo(Try-Sar-Sar-Lys(DNS)-Pro) is located near the polar head groups of lipid molecules and the pre-

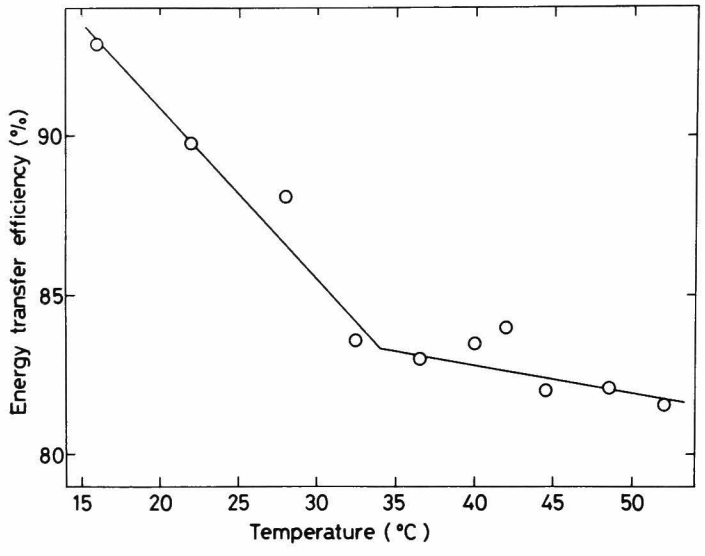

Fig. 3. The temperature dependence of intramolecular energy transfer of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in DPPC liposome. (cyclic peptide) = 5.25×10^{-6} M.

transition of membrane makes the orientation of indolyl and dansyl groups favorable for energy transfer. The present result is an example showing that the structural change of membrane by the phase transition affects the conformation of membrane peptide.

Control of the Intramolecular Energy Transfer by Complexation with Metal Ion

The function of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) is two-fold; it forms a complex with metal ions and it

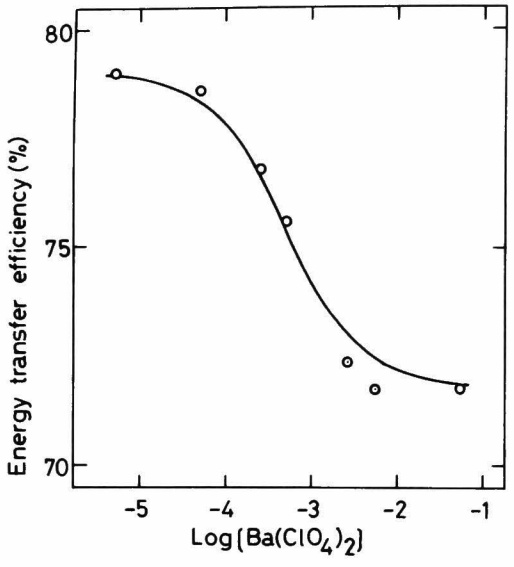

Fig. 4. Dependence of intramolecular energy transfer of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) on the concentration of Ba^{2+} in CH_3OH/H_2O (95/5 v/v). For the calculation of the theoretical value (shown by the curve), see the text. (cyclic peptide) = 5.25 x 10^{-6} M.

undergoes an intramolecular energy transfer. Figure 4 shows the change of the intramolecular energy-transfer efficiency with the addition of Ba^{2+}. The energy-transfer efficiency begins to decrease by the addition of about 10^{-4} M Ba^{2+} and ultimately comes to about 72% at 10^{-2} M Ba^{2+}. The theoretical efficiency of energy transfer was calculated as follows. The efficiencies of energy transfer of the cyclic pentapeptide were taken as 79%

for the metal ion-free state and 71.8% for the complexed state. The fractions of free and complexed cyclic peptides at various Ba^{2+} concentrations were calculated using the equilibrium constant obtained from cd spectra. The energy-transfer efficiencies at various Ba^{2+} thus calculated are shown in Figure 4. The observed values are in good agreement with the calculated curve. Therefore, the decrease of the intramolecular energy-transfer efficiency by Ba^{2+} can be ascribed to the complex formation of the cyclic peptide with Ba^{2+}. The following observation supports this account that the addition of Ba^{2+} to the 95% aqueous methanol solution of cyclo(Try-Sar-Sar-Lys(Z)-Pro) caused a blue shift of the maximum fluorescence and a slight increase of the quantum yield. The critical distance of the energy transfer calculated on the basis of the fluorescence spectrum was almost the same as that in the absence of Ba^{2+}. Therefore, the decrease of the energy-transfer efficiency due to the complex formation with Ba^{2+} should be ascribed to a different orientation of the chromophores. The conformational change of the ring structure by complexation should be responsible for that.

The effect of conformational change upon the intramolecular energy-transfer efficiency was not so large. The conformational change of the cyclic skeleton should not seriously affect the orientation of chromophores, because the dansyl group is bound to the end of a very flexible side chain.

A cyclic peptide, in which an energy donor and an acceptor are located close to the cyclic skeleton and the distance between them is close to the critical distance of energy transfer should be very interesting, because with this type of cyclic peptide a remarkable

change of the intramolecular energy-transfer efficiency with complexation is expected. In this case the energy-transfer efficiency might also be controlled by the change of membrane structure.

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ABSTRACT

PART I SYNTHESIS AND CONFORMATION OF CYCLIC PEPTIDES

Chapter 1 Synthesis and Conformation of Cyclic Hexapeptide Cyclo(Pro-Sar-Sar)$_2$

A cyclic hexapeptide, cyclo(Pro-Sar-Sar)$_2$, which consists of N-substituted amino acids only was synthesized. Seven different C_2-symmetric conformations due to the cis/trans isomerization of N-substituted peptide bonds were detected in organic solvents, which were distinguishable from each other on the nmr time scale. Allowed C_2-symmetric conformations were computed on the basis of a hard-sphere model. Some conformations detected in nmr spectra were not allowed in the calculation. This disagreement suggests that some asymmetric conformations with regard to the rotation around single bonds are averaged out due to a rapid rotation on the nmr time scale. The flexibility of cyclo(Pro-Sar-Sar)$_2$ was discussed in terms of the thermodynamic and the kinetic senses.

Chapter 2 Synthesis and Conformation of Cyclic Pentapeptide Containing a Tryptophan Residue

Cyclic pentapeptide containing an energy donor group and an energy acceptor group within a molecule, cyclo(Try-Sar-Sar-Lys(DNS)-Pro), was synthesized as an useful cyclic peptide for the fluorescence analysis in lipid membrane. To avoid the oxidation and other side reactions of tryptophan residue during the synthesis,

the indolyl group was protected by a formyl group. The conformational properties were investigated mainly on a precursor, cyclo(Try-Sar-Sar-Lys(Z)-Pro). This cyclic pentapeptide was apt to take a conformation containing one cis sarcosyl peptide bond and an all-trans β-turn structure in which the Pro residue takes the 3rd position in chloroform and acetonitrile, which is the first example found in synthetic peptides of L-L sequence.

Chapter 3 Synthesis and Conformation of Cyclic Octapeptides, Cyclo(Phe-Pro)$_4$, Cyclo(Leu-Pro)$_4$, and cyclo(Lys(Z)-Pro)$_4$

Cyclic octapeptides, cyclo(X-Pro)$_4$, where X represents Phe, Leu or Lys(Z), were synthesized for the first examples of all-L cyclic octapeptide. C_2-symmetric conformer containing two cis peptide bonds was always found in all these cyclic octapeptides in organic solvents. The C_2-symmetric conformation of cyclo(Phe-Pro)$_4$ contained a β-turn structure in which a Pro residue takes the 3rd position and a cis peptide bond is involved, which is the first example of such conformation found in synthetic peptides. The numbers of available conformations due to the cis/trans isomerization of Pro peptide bonds depended on the nature of solvent and X residue; they decreased in the order cyclo(Lys(Z)-Pro)$_4$ > cyclo-(Leu-Pro)$_4$ > cyclo(Phe-Pro)$_4$ in $CDCl_3$. ^{13}C spin-lattice relaxation times of these cyclic octapeptides were measured, and the contribution of segmental mobility to T_1 was found to vary with the nature of X residue. These conformational properties of cyclic octapeptides were discussed in terms of the steric repulsion between the side chains.

PART II INTERACTIONS OF CYCLIC PEPTIDES WITH SMALL MOLECULES

Chapter 4 Synthesis, Conformation, and Interactions with Small Molecules of Bis(Cyclic Dipeptides)

Bis(cyclic dipeptides), cyclo(Lys-Pro) cyclo(Glu-Pro) and S,S'-bis(cyclo(hemiCys-Pro)), were synthesized. These bis(cyclic dipeptides) formed complexes very efficiently with Ba^{2+} and Na^{+} in ethanol solution because of the intramolecular cooperation of two cyclic dipeptide moieties, the bis-effect. Cyclo(Lys-Pro) cyclo-(Glu-Pro) stacked sodium 8-anilino-1-naphthalenesulfonate in aqueous solution. The complexation behaviors were controlled by the nature of the bridge connecting two cyclic dipeptide moieties. The geometry of the complex between S,S'-bis(cyclo(hemiCys-Pro)) and metal ion was analyzed with the help of various spectroscopic measurements.

Chapter 5 Interactions of Cyclic Hexapeptide Cyclo(Pro-Sar-Sar)$_2$ with Small Molecules in Organic Solvents

Cyclo(Pro-Sar-Sar)$_2$ was found to form complexes with Li^{+}, K^{+}, Ba^{2+}, and Cu^{2+} in ethanol solution accompanying a conformational change into a single conformer. The conformation of cyclo(Pro-Sar-Sar)$_2$ in the Li^{+}-complex was different from that in the Cu^{2+}-complex. These findings indicate the conformational flexibility of cyclo(Pro-Sar-Sar)$_2$, which allows the adjustment of conformation according to the size and the shape of guest molecule. The equilibrium constant

for the complexation with Li^+ in ethanol was 2.3×10^2 M^{-1}, and cyclo(Pro-Sar-Sar)$_2$ in the complex took an asymmetric conformation. The addition of α-amino acid ester hydrochloride to dioxane/ethanol (4/5 v/v) solution of cyclo(Pro-Sar-Sar)$_2$ caused the conformational change of cyclo(Pro-Sar-Sar)$_2$ as well, but in this case it did not converge into a single conformation because of weak interaction. The interaction was strengthened with aromatic α-amino acid ester hydrochloride due to the aromatic-amide interactions. The rate of exchange between unbound α-amino acid ester hydrochlorides and those complexed with cyclo(Pro-Sar-Sar)$_2$ was dependent on the nature of α-amino acid.

Chapter 6 Interactions of Cyclic Octapeptides, Cyclo(Phe-Pro)$_4$, Cyclo(Leu-Pro)$_4$, and Cyclo(Lys(Z)-Pro)$_4$, with Small Molecules in Organic Solvents

In alcoholic solution, cyclo(Phe-Pro)$_4$ did not form complexes with metal ions, whereas cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ formed complexes selectively with Ba^{2+} and Ca^{2+} ions. The relation between the complexation and the flexibility of the cyclic octapeptides was discussed. Changing the solvent from alcohol to acetonitrile, the complexation behavior was greatly different. In acetonitrile cyclo(Phe-Pro)$_4$ was found to form a complex with Ba^{2+}, and cd spectra of cyclo(Leu-Pro)$_4$ and cyclo(Lys(Z)-Pro)$_4$ changed sharply upon complexation with K^+. Rate constants of the complex formation between the cyclic octapeptides and metal salts were in the range of $0.7 \sim 12$ $M^{-1} \cdot min^{-1}$ in alcoholic solution. One of the two kinds of complex

formations in acetonitrile was much faster than that in alcoholic solution.

PART III MEMBRANE ACTIVITIES OF OLIGOPEPTIDES

Chapter 7 Ion Transport through Liquid Membrane by Synthetic Cyclic Octapeptides

Synthetic cyclic octapeptides, cyclo(Leu-Pro)$_4$, cyclo(Phe-Pro)$_4$, and cyclo(Lys(Z)-Pro)$_4$, transported K^+ and Ba^{2+} through a chloroform liquid membrane. The rate of cation transport was correlated with the ability of cation extraction from aqueous phase to chloroform phase. Among them cyclo(Leu-Pro)$_4$ was most efficient and transported K^+ and Ba^{2+} selectively among other alkali and alkaline earth cations, respectively. The rate of K^+ transport by cyclo(Leu-Pro)$_4$ was about 1/3 as fast as that by dicyclohexyl-18-crown-6. The rate determining step of these cation transports through liquid membrane is considered to be the diffusion step through the boundary layer. A transport of picrate anion against its concentration gradient was realized by cyclo(Leu-Pro)$_4$, which was coupled with the selective transport of K^+. Complex formation in liposome between cyclo(Leu-Pro)$_4$ and Ba^{2+} was observed, but the binding constant was low. To generate the membrane potential according to the difference of caion concentration across the lipid membrane, it was suggested that a fast conformational change is required for the cyclic peptide.

Chapter 8 Interactions of Peptides in the Assembly of Lipid Molecules by Fluorescence Behavior

In order to get general informations about the peptide-lipid and the peptide-peptide interaction in liposome, behaviors of hydrophobic linear dipeptides containing tryptophan in liposome were investigated by the fluorescence method. The linear dipeptides were buried into the hydrophobic region of liposome to induce a blue shift of the fluorescence. With the addition of various anthracene derivatives to liposome containing the dipeptides, the energy transfer from Try group to anthryl group took place, which increased as the temperature decreased below the phase-transition temperature of the membrane. This phenomenon was explained in terms of the phase separation of the membrane, in which crystalline regions without the probes and the domains containing high concentrations of probes were intermixed. The energy-transfer efficiency was larger for peptide acceptors than for lipid acceptors. This suggests that special ineractions should exist between donor and acceptor in liposome when both are peptides.

Chapter 9 Intramolecular Transfer of Excitation Energy, Complexation with Metal Ions, and Interactions with Liposome of Cyclic Pentapeptide Containing a Tryptophan Residue

The synthetic cyclic pentapeptide, cyclo(Try-Sar-Sar-Lys(DNS)-Pro), is provided with two functions;

one is the selective complex formation with Ba^{2+} ion and the other is the energy acceptance and its intramolecular transfer. The correlation between the two functionalities of cyclic pentapeptide was investigated in 95% methanol, and the intramolecular energy-transfer efficiency of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) was found to decrease with complexation with Ba^{2+}. The cyclic pentapeptide was shown to interact with liposome consisting of dipalmitoylphosphatidylcholine. The intramolecular energy-transfer efficiency of cyclo(Try-Sar-Sar-Lys(DNS)-Pro) in ethanol was almost constant between -60°C and 40°C, while in liposome it increased at lower temperatures than 34°C as a result of the pre-transition of the lipid assembly.

LIST OF PUBLICATIONS

Chapter 1	Int. J. Biol. Macromol., 3, 183 (1981).
Chapter 2	Helv. Chim. Acta, submitted.
Chapter 3	Biopolymers, submitted.
Chapter 4	Helv. Chim. Acta, 65, 775 (1982).
Chapter 5	Int. J. Biol. Macromol., 3, 225 (1981).
Chapter 6	Biopolymers, submitted.
Chapter 7	Biopolymers, submitted.
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